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Cycles of Ubiquitination and Deubiquitination Critically Regulate Growth Factor-Mediated Activation of Akt Signaling

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Abstract

K63-linked ubiquitination of Akt is a posttranslational modification that plays a critical role in growth factor-mediated membrane recruitment and activation of Akt. Although E3 ligases involved in growth factor-induced Akt ubiquitination have been defined, the deubiquitinating enzyme (DUB) that triggers deubiquitination of Akt and the function of Akt deubiquitination remain largely unclear. Here, we showed that CYLD was a DUB for Akt and suppressed growth factor-mediated Akt ubiquitination and activation. CYLD directly removed ubiquitin moieties on Akt under serum-starved conditions. CYLD dissociated from Akt upon growth factor stimulation, thereby allowing E3 ligases to induce ubiquitination and activation of Akt. CYLD deficiency also promoted cancer cell proliferation, survival, glucose uptake and growth of prostate tumors. Our findings reveal the crucial role of cycles of ubiquitination and deubiquitination of Akt in its membrane recruitment and activation, and further identifies CYLD as a molecular switch for these processes.

INTRODUCTION

Akt (also known as protein kinase B) kinase plays a central role in cell proliferation, survival, metabolism and tumorigenesis (1–3). Akt normally resides in the cytosol under serum-starved conditions, but translocates to the plasma membrane where it is subsequently

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phosphorylated and activated in response to growth factor treatment. Phosphorylation of Akt at Thr³⁰⁸ by phosphoinositide-dependent kinase-1 (PDK1) and at Ser⁴⁷³ by mammalian target of rapamycin (mTOR) complex 2 (mTORC2) is required for full Akt activation (3, 4). Phosphorylation and activation of Akt requires anchoring to the plasma membrane by the signaling lipid phosphoinositol (3,4,5) triphosphate (PIP₃), which is generated by phosphoinositide 3-kinase (PI3K) (1–3), and K63-linked ubiquitination of Akt, which is induced by growth factors (3, 5–7). Distinct E3 ligases mediate ubiquitination and activation of Akt by different growth factors (5). In particular, the E3 ligase TNF receptor-associated factor 6 (TRAF6) selectively drives ubiquitination and activation of Akt in response to insulin-like growth factor-1 (IGF-1) stimulation, whereas the E3 ligase S-phase kinase-associated protein 2 (Skp2) SCF is involved in these events upon epidermal growth factor (EGF) treatment (5). Akt is kept in a hypoubiquitinated state under serum-starved conditions, and undergoes K63-linked ubiquitination in response to growth factor treatment (5, 6). One outstanding question is how ubiquitination and deubiquitination cycles of Akt are regulated in response to distinct culture conditions. We hypothesized that deubiquitinating enzymes (DUBs) in cells may serve as molecular switches for this process by sensing the concentration of growth factors.

In this study, we sought to understand the function of deubiquitination of Akt and its regulation by identifying DUBs for Akt. We show that cylindromatosis (CYLD) is a direct DUB for Akt and suppresses growth factor-mediated ubiquitination, membrane recruitment and activation of Akt in response to growth factor stimulation.

RESULTS

CYLD is a DUB for Akt

To understand the mechanism by which Akt deubiquitination process is regulated, we screened a panel of potential DUBs for Akt by performing deubiquitination assays in cells ectopically expressing DUBs. Among the DUBs, only CYLD effectively reduced the ubiquitination of Akt (Fig. 1A and fig. S1). Because Akt has three isoforms (Akt1, Akt2 and Akt3), we next examined whether CYLD displays differential specificity for various Akt isoforms and found that CYLD promoted deubiquitination of Akt1 and Akt2 (fig. S2A).

CYLD inhibits activation of nuclear factor- κ B (NF- κ B) and innate immune response (8–10). Mutation of the gene encoding CYLD is associated with some inherited diseases such as familial cylindromatosis (FC), which is characterized by the development of tumors from skin appendages (cylindromatosis) (9, 11). Furthermore, loss of CYLD is found in various human cancers, suggesting that CYLD may be a tumor suppressor (12). We found that the inhibitory effect of CYLD on ubiquitination of Akt depended on its catalytic activity, because the catalytically dead mutant of CYLD (Cys⁶⁰¹→Ala⁶⁰¹; C601A) failed to attenuate ubiquitination of Akt (Fig. 1B). CYLD specifically removed K63-linked ubiquitination of Akt (Fig. 1C). Because TRAF6 and Skp2 are E3 ligases for Akt and play critical roles in Akt membrane recruitment, phosphorylation and activation (5, 6), we determined whether CYLD also regulates TRAF6 or Skp2-mediated ubiquitination of Akt. Wild-type CYLD, but not its catalytic mutant, inhibited both TRAF6 and Skp2-mediated Akt ubiquitination (Fig. 1B, D). To determine whether CYLD is a direct DUB for Akt, we performed *in vitro* deubiquitination assays and found that deubiquitination of Akt was mediated by wild-type CYLD but not the C601A mutant (Fig. 1E). These results suggest that CYLD is a *bona fide* DUB for Akt.

CLYD interacts with Akt and its deficiency induces basal Akt ubiquitination

We next determined whether CYLD deficiency promotes ubiquitination of endogenous Akt in response to IGF-1 stimulation. In wild-type primary MEFs, endogenous Akt was basally ubiquitinated under serum-starved conditions and ubiquitination was increased by IGF-1, consistent with our previous report (Fig. 2A) (6). Ubiquitination of endogenous Akt under serum-starved condition in *Cyld*^{-/-} MEFs was similar to that seen in wild-type MEFs treated with IGF-1, but was not further increased by IGF-1 treatment (Fig. 2A). Similarly, prostate cancer cells lacking CYLD also displayed increased basal ubiquitination of Akt in the absence of IGF-1 treatment (Fig. 2B). These results suggest that CYLD keeps Akt in the hypoubiquitinated state and that its deficiency facilitates ubiquitination of Akt.

We next determined whether Akt associates with CYLD. Co-immunoprecipitation assays showed that exogenously expressed Akt1 interacted with both wild-type and mutant CYLD (C601A) (Fig. 2C). Likewise, exogenously expressed Akt2 and Akt3 also interacted with CYLD (fig. S2B). Notably, phosphorylation of Akt at Thr³⁰⁸ and Ser⁴⁷³, which indicate activation of Akt, was attenuated by wild-type CYLD, but not C601A CYLD (Fig. 2C). Moreover, we found that endogenous Akt interacted with CYLD under serum-starved conditions in a reciprocal immunoprecipitation assay (Fig. 2D). The interaction between CYLD and Akt was lost after 15 minutes of IGF-1 treatment (Fig. 2E), whereas the interaction between Akt and TRAF6 was induced at the same time point (6), suggesting that CYLD and TRAF6 may compete with each other for Akt binding. Indeed, we found that TRAF6 overexpression inhibited the binding of CYLD to Akt in a dose-dependent manner (Fig. 2F). These results suggest that CYLD interacts with Akt under serum starvation conditions and dissociates from Akt upon growth factor stimulation, which may allow E3 ligases to bind to and ubiquitinate Akt.

The interaction of Akt with CYLD is independent of its ubiquitination or phosphorylation status

We next examined whether the ubiquitination status of Akt affects its interaction with CYLD. CYLD bound to both non-ubiquitinated and ubiquitinated Akt forms (Fig. 3A), and both wild-type Akt and the ubiquitination-deficient K8R mutant (6) interacted with CYLD (Fig. 3B). Because IGF-1 disrupts the interaction between Akt and CYLD, we also determined whether phosphorylation of Akt attenuated (or disrupted) its interaction with CYLD. Both wild-type and the constitutively active Akt mutant T308D/S473D (Akt-DD) interacted with CYLD (fig. S3A), suggesting that the phosphorylation of Akt does not affect Akt and CYLD interaction. Consistent with this notion, the phosphorylation defective Akt mutant T308A/T450A/S473A (Akt-AAA) also bound to CYLD, and overexpression of TRAF6 induced a dose-dependent decrease in the interaction between CYLD and the Akt-AAA mutant (fig. S3B). These results suggest that the ubiquitination or phosphorylation status of Akt may not be critical for its interaction with CYLD.

Ubiquitination and deubiquitination of Akt is independent of PI3K activity

PI3K activity plays an important role in growth factor-induced activation of Akt. To determine whether PI3K is involved in ubiquitination and deubiquitination of Akt, we performed cellular ubiquitination and deubiquitination assays for endogenous Akt in the presence or absence of the PI3K inhibitor LY294002. Inhibition of PI3K activity did not inhibit IGF-1-induced ubiquitination and CYLD-mediated deubiquitination of Akt (Fig. 3C, D).

We further determined whether polyubiquitination-mediated membrane recruitment of Akt could lead to its activation without PI3K activity. To this end, HEK293T cells were cotransfected with wild-type Akt, ubiquitination-deficient K8R Akt mutant or cancer-

associated E17K Akt mutant which displays much higher ubiquitination of Akt compared to wild-type Akt (6) in the presence or absence of LY294002. Consistent with our previous observation (6), we found that phosphorylation of Akt was increased in cells expressing the E17K mutant, whereas those expressing the ubiquitination deficient K8R mutant showed reduced phosphorylation of Akt compared to wild-type Akt. However, PI3K inhibition by LY294002 reduced phosphorylation of wild-type Akt and the E17K and K8R mutants (Fig. 3E), suggesting that although polyubiquitination of Akt is required for its phosphorylation and activation, it is not sufficient to lead to its activation without PI3K activation.

CYLD deficiency promotes membrane recruitment and phosphorylation of Akt

The finding that CYLD interacts with Akt and suppresses ubiquitination of Akt prompted us to determine whether CYLD prevents phosphorylation of Akt in response to growth factor stimulation. Indeed, *Cyld*^{-/-} MEFs displayed higher basal phosphorylation of Akt (at 0 min) and slightly enhanced IGF-1 mediated phosphorylation of Akt compared to wild-type MEFs (Fig. 4A), which correlated with the increased basal ubiquitination of Akt caused by *Cyld* deficiency (Fig. 2A). Likewise, CYLD knockdown also promoted basal and IGF-1 and EGF-induced phosphorylation of Akt in prostate cancer cell lines (Fig. 4B, C). Conversely, restoration of wild-type CYLD, but not the C601A mutant, into CYLD knockdown cancer cells reversed IGF-1-induced phosphorylation of Akt, suggesting that the enzymatic activity of CYLD plays a critical role in suppressing growth factor-induced phosphorylation of Akt (Fig. 4D). Accordingly, our results suggest that CYLD opposes ubiquitination of Akt and thereby inhibits its phosphorylation in response to stimulation with growth factors.

We have shown that ubiquitination of Akt is critical for growth factor-mediated membrane recruitment and activation of Akt (6). Given that CYLD opposes ubiquitination of Akt, it is possible that CYLD prevents membrane recruitment of Akt, in turn inhibits its phosphorylation and activation. To test this hypothesis, we performed biochemical fractionation experiments to determine whether CYLD regulates membrane recruitment of Akt in the presence or absence of IGF-1 and EGF treatment. Consistent with this notion, either *Cyld* deficiency or CYLD knockdown enhanced basal membrane recruitment of Akt as well as basal and IGF-1 and EGF-mediated phosphorylation of Akt in the plasma membrane (Fig. 4E–G). Similar results were also obtained from immunofluorescent analysis of wild-type and *Cyld*^{-/-} MEFs (fig. S4A, B). We next determined whether PI3K activity is required for membrane translocation of Akt in wild-type and *Cyld*^{-/-} MEFs. PI3K inhibition efficiently blocked IGF-1-induced membrane translocation of Akt in both wild-type and *Cyld*^{-/-} MEFs, but failed to inhibit constitutive membrane recruitment of Akt in *Cyld*^{-/-} MEFs under serum-starved conditions (fig. S5A, B). Thus, our results support the notion that CYLD opposes Akt ubiquitination, in turn inhibiting growth factor-mediated membrane recruitment and phosphorylation of Akt.

CYLD deficiency promotes cancer cell proliferation, cell survival and tumorigenesis

Akt plays a crucial role in various biological processes, such as cell proliferation, cell survival and metabolism, which in turn promotes tumorigenesis. Because CYLD suppresses ubiquitination and activation of Akt, it is possible that CYLD may also inhibit cancer cell proliferation and survival. In support of this notion, we found that prostate cancer cells with CYLD knockdown proliferated faster than control knockdown cancer cells (Fig. 5A). Similar results were also obtained from *Cyld*^{-/-} MEFs and a breast cancer cell line with CYLD knockdown (fig. S6A, B). In addition, CYLD-deficient prostate cancer cells showed enhanced cell survival in response to treatment with the apoptosis inducer cisplatin (Fig. 5B).

To determine whether CYLD inhibits cell proliferation and survival by suppressing activation of Akt, we performed cell proliferation and apoptosis assays on control and CYLD-knockdown prostate cancer cells treated with or without the PI3K inhibitors LY294002 or wortmannin. PI3K inhibitors compromised the increase in cell proliferation and survival in CYLD deficient prostate cancer cells, suggesting that CYLD inhibits these biological events through the PI3K-Akt pathway (Fig. 5C, D). However, because the PI3K inhibitors did not completely abolish the effect of CYLD deficiency on cell proliferation and survival, we cannot exclude the possibility that CYLD may also regulate other pathways. Because CYLD inhibits NF- κ B activation (8, 10, 11), it is probable that both PI3K-Akt and NF- κ B signaling pathways are involved in CYLD-regulated cell proliferation and survival.

Akt promotes glucose uptake for glycolysis and ATP production by inducing the plasma membrane localization of glucose transporters, such as Glut1 and Glut4 (13–16). Because CYLD attenuates Akt activation, we hypothesized that CYLD may inhibit membrane translocation of both Glut1 and Glut4 and glucose uptake. Indeed, we found that *Cyld*^{-/-} MEFs showed increased membrane localization of Glut1 and Glut4 compared to wild-type MEFs (Fig. 5E). Although glucose uptake in *Cyld*^{-/-} MEFs was slightly higher than in wild-type MEFs, this difference was not statistically significant (Fig. 5F). However, CYLD knockdown prostate cancer cells displayed increased membrane localization of Glut1, but not of Glut4, and higher glucose uptake compared to control knockdown cells (Fig. 5E, F). Accordingly, these results suggest that CYLD inhibits proliferation and survival of prostate cancer cells, membrane translocation of Glut1, and glucose uptake.

Loss of CYLD is found in melanoma, colon cancer and liver cancer (12). To determine whether CYLD also plays a tumor suppressive role in prostate cancer, we subcutaneously injected prostate cancer lines with control or CYLD knockdown into athymic nude mice and monitored tumor growth. We found that CYLD knockdown in two different prostate cancer cell lines promoted prostate tumor growth (Fig. 6A). We next investigated whether there was an inverse relationship between CYLD and Akt activation in human prostate cancer samples. We retrospectively analyzed representative tissue blocks of 80 primary prostate cancers in individuals who underwent radical prostatectomy. Immunohistochemistry results showed a significant negative correlation between phosphorylation of Akt at Ser⁴⁷³ and CYLD abundance (Fig. 6B, C). In addition, compared to early stage prostate cancer samples (stage I-IIA), advanced stage prostate cancer samples (stage IIB-III) displayed increased phosphorylation of Akt at Ser⁴⁷³ and decreased CYLD abundance (Fig. 6D). These results suggest that CYLD suppresses Akt activation to inhibit prostate cancer cells growth and development. Taken together, our results underscore the role of CYLD in attenuating proliferation, glucose uptake, and survival of cancer cells, and thus in tumor suppression.

DISCUSSION

Our study provides insight into the regulation of the deubiquitination of Akt. We identified CYLD as a critical DUB that keeps Akt in a hypoubiquitinated and inactive form. As a result, loss of CYLD enhances basal ubiquitination, membrane recruitment and activation of Akt, thereby promoting cell proliferation, cell survival and glucose uptake, which are all important pro-tumorigenic events. During the preparation of our manuscript, Li and colleagues reported that CYLD inhibits TGF- β -mediated lung fibrosis by decreasing Smad3 stability and Akt ubiquitination, which supports our notion that CYLD is a DUB for Akt (17). However, it is unclear in their study whether CYLD inhibits growth factor-mediated ubiquitination, membrane recruitment and activation of Akt to promote tumorigenesis. We found that CYLD interacts with Akt under serum-free condition, but dissociates from Akt upon growth factor stimulation. However, the interaction between Akt and CYLD is independent of the ubiquitination and phosphorylation status of Akt. We also demonstrated

that PI3K activity, which is usually essential for growth factor-mediated Akt activation, is dispensable for growth factor-mediated ubiquitination and CYLD-mediated deubiquitination of Akt. Although PI3K is not required for these process, it is still critical for the phosphorylation of the ubiquitin-mimetic E17K mutant.

Our study demonstrates the role of CYLD in growth factor-mediated ubiquitination, membrane recruitment and activation of Akt. Moreover, we also unravel several new functions of CYLD in inhibiting membrane translocation of Glut1, glucose uptake and prostate cancer development, thereby offering new paradigms for prostate cancer treatment. Although CYLD abundance is decreased in colon cancer, liver cancer and melanoma (12), its function in prostate cancer development was previously unknown. In our study, we demonstrated that CYLD deficiency promotes prostate cancer progression in a xenograft mouse model, and immunohistochemistry results revealed that CYLD abundance is reduced in advanced stage of prostate cancer samples and inversely correlates with Akt activation. These results suggest that CYLD may play a tumor suppressive role in prostate cancer development by inhibiting oncogenic Akt signaling.

On the basis of our findings, we propose a working model by which growth factors regulate ubiquitination, membrane recruitment and activation of Akt (Fig. 7). CYLD interacts with and keeps Akt in a hypoubiquitinated and inactive stage by directly removing Akt ubiquitination under serum-starvation conditions. Upon stimulation with growth factors, CYLD dissociates from Akt, which then allows E3 ligases such as TRAF6 and Skp2 to interact with Akt and ubiquitinate Akt for subsequent membrane recruitment and activation. Thus, our study suggests that CYLD serves as a critical switch to orchestrate the ubiquitination and deubiquitination cycles of Akt, thereby regulating membrane recruitment, activation and tumorigenesis of Akt.

MATERIALS AND METHODS

Mice, cell cultures and reagents

Mouse embryonic fibroblasts (MEFs) from *Cyld*^{+/+} and *Cyld*^{-/-} mice were prepared as previously described (18–21). MEFs, PC-3, DU-145, MDA-MB-231 and HEK293T cells were cultured in DMEM containing 10% FBS. Flag-HA-USP1, Flag-HA-USP3, Flag-HA-USP5, Flag-HA-USP8, Flag-HA-USP10, Flag-HA-USP21, Flag-HA-USP26, were described previously (22). Myc-USP7 was obtained from Dr. Pier Paolo Pandolfi (23). Flag-USP22 was obtained from Dr. Didier Devys. (His)₆-ubiquitin and HA-Akt1, Flag-TRAF6 and Xp-Skp2 pGEX-4X1-TRAF6, and pGEX-4X1-Akt, HA-Ub-K48 and HA-Ub-K63 constructs have been described previously (6, 20). Flag-CYLD and Flag-CYLD-C/A (C601A) were obtained from Dr. Xin. Lin. Human recombinant IGF-1, EGF and N-Ethylmaleimide were obtained from Calbiochem. Cisplatin was purchased from Alexis Biochemicals. The PI3K inhibitors LY294002 and wortmannin were purchased from Cell Signaling.

Immunoprecipitation and immunoblotting

Immunoprecipitation and immunoblotting were performed essentially as described elsewhere (6, 20). For protein-protein interactions, cells were lysed by E1A lysis buffer [250 mM NaCl, 50 mM HEPES (pH 7.5), 0.1% NP-40, 5 mM EDTA and protease inhibitor cocktail (Roche)]. The following antibodies were used for immunoprecipitation and immunoblotting: anti-CYLD antibody (IP: 1:100, gift from Dr. Shao-Cong Sun; IB: 1:1000, Santa Cruz), anti-ubiquitin antibody (IB: 1:1000, Santa Cruz), anti-N-cadherin (IB: 1:1000, Santa Cruz), anti-pan-Akt antibody (IP: 1:200; IB: 1:1000, Cell Signaling), anti-phospho (Ser⁴⁷³)-Akt antibody (IB: 1:2000, Cell Signaling), anti-phospho (Thr³⁰⁸)-Akt (IB: 1:1000,

Cell Signaling), anti-Glut1 antibody (IB: 1:1000, Abcam), anti-Glut4 antibody (IB: 1:1000, Millipore), anti- α -tubulin antibody (IB: 1:1000, Sigma), anti- β -actin antibody (IB: 1:1000, Sigma), anti-HA antibody (IB: 1:10000, Covance) and anti-Flag antibody (M2, IP: 1:200; IB: 1:3000, Sigma).

Cellular ubiquitination assays

Cellular ubiquitination assays were performed as previously described (6). Briefly, HEK293T cells were cotransfected with the indicated plasmids for 48 hours and lysed by denaturing buffer (6 M guanidine-HCl, 0.1 M Na₂HPO₄/NaH₂PO₄, 10 mM imidazole), followed by nickel bead purification and immunoblot analysis. For endogenous ubiquitination assays, *Cyld*^{+/+} and *Cyld*^{-/-} MEFs or PC-3 cells with control and CYLD knockdown were seeded in 100 mm² dishes and were serum-starved for 24 hours in DMEM containing 0.1% FBS. After starvation, cells were treated with or without 50 ng/ml IGF-1 for 15 min. Cells were lysed by RIPA lysis buffer [50 mM TrisHCl pH7.4, 150 mM NaCl, 2 mM EDTA, 1% NP-40, 0.1% SDS and protease inhibitor cocktail (Roche)] with 10 mM N-Ethylmaleimide (Calbiochem). Immunoprecipitation was performed to pull-down polyubiquitinated Akt proteins. Samples were subjected to SDS-PAGE and immunoblotted with anti-ubiquitin antibody.

In vitro deubiquitination assay

To purify ubiquitinated Akt (Akt-Ub), HEK293T cells were transfected with HA-Akt along with His-Ub. After 48 hours, the cells were lysed with RIPA buffer (1% NP-40) and immunoprecipitated with anti-HA antibody. Purified Akt-Ub was washed by RIPA buffer for 4 times. To purify CYLD, HEK293T cells were transfected with Flag-CYLD-WT or Flag-CYLD-C/A for 48 hours and lysed with Flag lysis buffer. The cell extracts were subjected to immunoprecipitation using anti-Flag antibody, followed by elution with Flag peptide. To perform *in vitro* ubiquitination assays, the purified Akt-Ub was incubated with purified CYLD-WT or CYLD-C/A in a deubiquitination buffer (50 mM Tris-HCl [pH 8.0], 50 mM NaCl, 1 mM EDTA, 10 mM DTT and 5% glycerol) for 2 hours at 37°C. After incubation, proteins on the beads were eluted in SDS-sample buffer, subjected to SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted with anti-Akt antibody.

Viral infection

For lentiviral short hairpin RNA (shRNA) infection, HEK293T cells were cotransfected control or CYLD shRNA with packing plasmids (deltaVPR8.9) and envelope plasmid (VSV-G) by using calcium phosphate precipitation technique. CYLD-lentiviral shRNA #1 (5'- TACTTAGACTCAACCTTATTC-3'), CYLD-lentiviral shRNA #2 (5'- AAGAAGGTCGTGGTCAAGGTC-3'), control shRNA (5'- GCAAGCTGACCCTGAAGTTC-3') were transfected with packing plasmids into HEK293T cells for 2 days, and virus particles containing CYLD or control shRNAs were used to infect PC-3, DU-145 and MDA-MB-231 cells. All the infected cells were cultured in medium containing 2 μ g/ml puromycin for selection up to 1 week.

Membrane fractionation assay

Cyld^{+/+} and *Cyld*^{-/-} MEFs or PC-3 cells with control and CYLD knockdown were seeded in 100 mm² dishes and were serum-starved for 24 hours in DMEM containing 0.1% FBS. After starvation, cells were treated with 50 ng/ml IGF-1 in indicated times. Cytosolic and membrane fractions were prepared using the ProteoExtract kit (Calbiochem) according to the manufacturer's standard procedures.

Cell Growth Assay

5×10^3 of *Cyld*^{+/+} and *Cyld*^{-/-} MEFs, 5×10^3 of DU-145 or MDA-MB-231 cells with control and CYLD knockdown, or 2×10^3 PC-3 cells with control and CYLD knockdown were seeded in 12 wells plate in triplicate. Cells were harvested, stained with trypan blue and counted viable cells every two days up to six days. Viable cells were counted on hemocytometer directly under the phase-contrast microscope.

Apoptosis assay

PC-3 or DU-145 cells with control and CYLD knockdown were seeded in 60 mm² dishes in triplicate. Cells were treated with/without 50 μ M Cisplatin for 24 hours, and cells were collected and labeled with Annexin V-FITC apoptosis detection kit (BD Pharmingen) according to the manufacturer's standard procedures, followed by flow cytometry analysis.

Glucose uptake assay

Cyld^{+/+} and *Cyld*^{-/-} MEFs or PC-3 cells with control and CYLD knockdown were seeded in 60 mm² dishes in triplicate. After 24 hours, cells were refreshed with 0.1% FBS and glucose-free DMEM for 24 hours serum-starvation. Cells treated with/without 50 ng/ml IGF-1 were grown in the presence of fluorescent glucose analog 2-NBDG (50 μ M; Invitrogen) for 1 hour (for PC-3 cells) or 24 hours (for MEFs), respectively. 2-NBDG uptake ratio by cells was analyzed by FACS analysis.

In vivo tumorigenesis assay

2×10^6 PC-3 stable cells with control and CYLD knockdown or 5×10^6 DU-145 stable cells with control and CYLD knockdown mixed with matrigel (1:1) were subcutaneously injected into the left flank of 6-week-old athymic nude mice. Tumor sizes of mice were measured weekly by using a caliper, and tumor volume was determined by using the standard formula: $L \times W^2 \times 0.52$, where L is the longest diameter and W is the shortest diameter.

Immunohistochemistry and statistical analysis

The immunohistochemistry study, which was approved by Institutional Review Board (IRB10102-004), was performed on representative tissue blocks of 80 primary prostate cancers from individuals who underwent radical prostatectomy. The sections were heated for epitope retrieval and endogenous peroxidase activity quenched in 3% H₂O₂ and blocking of nonspecific immunoreactivity was performed by incubation with 10% normal horse serum. The primary antibodies were detected by using the DAKO EnVision kit (DAKO, Ely, United Kingdom) for Akt phosphorylated at Ser⁴⁷³ (1:50, Cell Signaling) and CYLD (1:100, Epitomics). To evaluate the abundance of both markers, the immunohistochemical signals were assessed using a combination of the percentage and intensity of positively stained tumor to generate a histological score (H-score) as previously described(24). Briefly, it was calculated using the following equation: $H\text{-score} = \sum P_i (i + 1)$, where i is the intensity (ranging from 0 to 4), and P_i is the percentage of stained tumor cells of each intensity, varying from 0% to 100%. The associations of H-score between these two markers and tumor stage were calculated by Spearman's correlation and Mann-Whitney U test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. CYLD is a DUB for Akt. (A) Cellular ubiquitination assays performed in HEK293T cells transfected with His-ubiquitin (His-Ub), hemagglutinin (HA)-Akt, along with various DUB constructs. Ni-nitrilotriacetic acid (Ni-NTA), nickel bead precipitate; WCE, whole-cell extracts. (B) Cellular ubiquitination assays performed in HEK293T cells transfected with HA-Akt, His-Ub and Flag-TRAF6, along with Flag-CYLD-WT or Flag-CYLD-C/A. C/A, enzyme-dead mutant (C601A). (C) Immunoblot analysis of Akt immunoprecipitates from HEK293T cells transfected with Akt, Flag-CYLD, along with HA-Ub K48 (K48-only ubiquitin) or HA-Ub K63 (K63-only ubiquitin). IB, immunoblot. IP, immunoprecipitation. (D) Cellular ubiquitination assays performed in HEK293T cells transfected with HA-Akt, His-Ub and Xp-Skp2, along with Flag-CYLD-WT or Flag-CYLD-C/A. (E) In vitro deubiquitination assays with purified ubiquitinated Akt proteins incubated with purified Flag-CYLD-C/A or Flag-CYLD-WT proteins. A representative blot from three independent experiments (N=3) is shown for each panel.

Fig. 2. CYLD interacts with Akt and inhibits the ubiquitination of endogenous Akt. (A) Immunoblot analysis of Akt immunoprecipitates from *Cyld*^{+/+} and *Cyld*^{-/-} MEFs that were serum-starved and treated with or without IGF-1. (B) Immunoblot analysis of Akt immunoprecipitates from control or CYLD stable knockdown PC-3 or DU-145 cells that were serum-starved and treated with or without IGF-1. (C) Immunoblot analysis of HA immunoprecipitates from HEK293T cells transfected with HA-Akt along with Flag-CYLD-WT or Flag-CYLD-C/A. (D) Immunoblot analysis of CYLD or Akt immunoprecipitates from serum-starved PC-3 cells. (E) Immunoblot analysis of Akt immunoprecipitates from PC-3 cells that were serum-starved and treated with IGF-1 at various time points. (F) Immunoblot analysis of HA immunoprecipitates from HEK293T cells transfected with HA-Akt along with Flag-CYLD or various amounts of Flag-TRAF6. A representative blot from three independent experiments is shown for each panel. The quantification of data for (E) and (F) are presented as the means ± S.D. of triplicate measurements in Table S1.

Fig. 3. PI3K activity is not required for ubiquitination and deubiquitination of Akt. (A) Immunoblot analysis of Flag immunoprecipitates from HEK293T cells transfected with Flag-CYLD, HA-Akt, His-Ub and TRAF6. (B) Immunoblot analysis of HA immunoprecipitates from HEK293T cells transfected with Flag-CYLD and His-Ub, along with HA-Akt-WT or HA-Akt K8R. (C) Immunoblot analysis of HA immunoprecipitates from serum-starved PC-3 cells that were pre-treated with or without the PI3K inhibitor LY294002 and treated with or without IGF-1. (D) Cellular ubiquitination assays performed in HEK293T cells transfected with HA-Akt, His-Ub, and Flag-CYLD that were pre-treated with or without LY294002. (E) Immunoblot analysis of HEK293T cells transfected with HA-Akt-WT, HA-Akt-E17K or HA-Akt-K8R that were treated with or without LY294002. A representative blot from three independent experiments (N=3) is shown for each panel.

Fig. 4. CYLD deficiency facilitates growth factor-mediated membrane recruitment and phosphorylation of Akt. (A) Immunoblot analysis of *Cyld*^{+/+} and *Cyld*^{-/-} MEFs that were serum-starved and treated with IGF-1 at various time points. (B) Immunoblot analysis of control or CYLD stable knockdown PC-3 or DU-145 cells that were serum-starved and treated with IGF-1 at various time points. (C) Immunoblot analysis of control or two different CYLD knockdown PC-3 cell lines that were serum-starved and treated with EGF at various time points. (D) Immunoblot analysis of CYLD knockdown PC-3 cells that were transfected with mock, Flag-CYLD-WT or Flag-CYLD-C/A, serum starved, and treated with IGF-1 at various time points. (E) Immunoblot analysis of membrane and cytosolic fractions of *Cyld*^{+/+} and *Cyld*^{-/-} MEFs that were serum-starved and treated with IGF-1 at various time points. (F) Immunoblot analysis of membrane and cytosolic fractions of control or CYLD stable knockdown PC-3 or DU-145 cells that were serum-starved and treated with IGF-1 at various time points. (G) Immunoblot analysis of membrane and cytosolic fractions of control or two CYLD knockdown PC-3 cell lines that were serum-starved and treated with EGF at various time points. A representative blot from three independent experiments (N=3) is shown for each panel. The quantification of data for (A), (B), (C) and (E) are presented as the means ± S.D. of triplicate measurements in Table S1.

Fig. 5. CYLD inhibits cancer cell proliferation, cell survival and glucose uptake. (A) Cell proliferation in control or CYLD stable knockdown PC-3 or DU-145 cells, presented as mean values $\pm$ S.D. from three biological replicates. $^{**}P < 0.01$, $^{***}P < 0.001$ for all pairwise comparisons by one-way ANOVA and post hoc intergroup comparisons with Sidak test. (B) Cell apoptosis as determined by Annexin V staining and flow cytometry analysis in control or CYLD stable knockdown PC-3 or DU-145 cells that were treated with vehicle or cisplatin. The result is presented as mean percentage values from three biological replicates. $^{*}P < 0.05$, $^{**}P < 0.01$ for all pairwise comparisons by Pearson chi-square test. (C) Cell proliferation in control or CYLD knockdown PC-3 or DU-145 cells treated with vehicle, LY294002 (LY) or wortmannin (Wort) respectively, presented as mean values $\pm$ S.D. from three biological replicates. $^{***}P < 0.001$ for all pairwise comparisons by one-way ANOVA and post hoc intergroup comparisons with Sidak test. (D) Cell apoptosis as determined as in (B) in control or CYLD stable knockdown PC-3 or DU-145 cells that were pretreated with vehicle, LY294002 (LY) or wortmannin (Wort), then with vehicle or cisplatin. The result is presented as mean percentage values from three biological replicates; $^{*}P < 0.05$, $^{**}P < 0.01$ for all pairwise comparisons by Pearson chi-square test. (E) Immunoblot analysis of Glut1

and Glut4 in cytosolic or membrane fractions of *Cyld*^{+/+} and *Cyld*^{-/-} MEFs or PC-3 cells with control or CYLD knockdown that were treated with IGF-1 for indicated time intervals. A representative blot from three independent experiments (N=3) is shown. (F) Analysis of glucose uptake ratios in *Cyld*^{+/+} and *Cyld*^{-/-} MEFs or PC-3 cells with control or CYLD knockdown treated with or without IGF-1 grown in the presence of the fluorescent glucose analog 2-NBDG. The result is presented as mean percentage values from three biological replicates. N.S., nonsignificant and ***P < 0.001 for all pairwise comparisons by Pearson chi-square test.

Fig. 6.

CYLD suppresses prostate cancer development. (A) PC-3 or DU-145 cells with control or CYLD knockdown were injected into nude mice (N=6 for each group) and the increase of tumor volume in mice was monitored every week. Results are presented as mean values $\pm$ S.D.; * $P < 0.05$ for all pairwise comparisons by one-way ANOVA and post hoc intergroup comparisons with Sidak test. (B) Immunohistochemistry in representative primary non-metastatic prostate cancer samples. A panel of images showing H&E staining, Akt phosphorylated at Ser⁴⁷³ and CYLD staining in early and advanced stage prostate cancer samples. Scale bar, 50 μ m. (C) Histological score (H-score) graph showing negative correlation between phosphorylation of Akt at Ser⁴⁷³ and CYLD abundance in prostate cancer samples. $P < 0.001$ by using Spearman's correlation and Mann-Whitney U test. (D) The associations of H-score between phosphorylation of Akt at Ser⁴⁷³ and CYLD abundance and different tumor stages. Results are presented as mean values $\pm$ S.D.; the P values were obtained with Spearman's correlation and Mann-Whitney U test.

Fig. 7. Model for growth factor-mediated ubiquitination and activation of Akt. In serum-starved cells (left panel), CYLD interacts with inactive Akt and keeps it in a hypoubiquitinated state. Growth factor signaling (dashed lines in right panel) may promote the dissociation of CYLD from Akt, thereby enabling the E3 ligases TRAF6 or Skp2 to interact with and ubiquitinate Akt, in turn facilitating its membrane recruitment, phosphorylation and activation. Activation of Akt therefore contributes to tumorigenesis by promoting cancer cell proliferation, survival and glucose metabolism.