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ORIGINAL ARTICLE

Akt is negatively regulated by the MULAN E3 ligase

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The serine/threonine kinase Akt functions in multiple cellular processes, including cell survival and tumor development. Studies of the mechanisms that negatively regulate Akt have focused on dephosphorylation-mediated inactivation. In this study, we identified a negative regulator of Akt, MULAN, which possesses both a RING finger domain and E3 ubiquitin ligase activity. Akt was found to directly interact with MULAN and to be ubiquitinated by MULAN *in vitro* and *in vivo*. Other molecular assays demonstrated that phosphorylated Akt is a substantive target for both interaction with MULAN and ubiquitination by MULAN. The results of the functional studies suggest that the degradation of Akt by MULAN suppresses cell proliferation and viability. These data provide insight into the Akt ubiquitination signaling network.

***Keywords*:** ubiquitin E3 ligase; inhibition; growth regulation

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Introduction

The serine/threonine kinase Akt (protein kinase B) is involved in the responses to essential cell stimuli, such as growth factors and insulin [1, 2]. Akt activation regulates multiple biological responses, including metabolism, cell proliferation, protein synthesis, cell growth, cell-cycle progression, and the suppression of apoptosis [3]. Akt is activated through platelet-derived growth factor, insulin, epidermal growth factor, basic fibroblast growth factor, and insulin-like growth factor I [2, 4, 5]. The downstream substrates of Akt have been identified at both the cellular and molecular levels. Glycogen synthase kinase 3 (GSK3), the first identified substrate for Akt, is negatively regulated through direct phosphorylation by Akt [6]. Akt inhibits GSK3 (especially GSK3β) activity, leading to β-catenin stabilization, nuclear localization, and target-gene activation [7-9].

Several regulators of Akt activation have been identified, which have provided critical information of the Akt regulatory signaling pathway. Phosphatidylinositol 3-kinase (PI3K) and phosphatase and tensin homolog (PTEN) are regulators of Akt [10]. PI3K induces Akt phosphorylation/activation, whereas PTEN inhibits Akt activation through the dephosphorylation of phosphatidylinositol-3,4,5-triphosphate, which is generated from phosphatidylinositol-4,5-bisphosphate by PI3K [2, 4, 11]. Negative regulators, such as C1 domain-containing PTEN, carboxy-terminal modulator protein, Trb3, and Keratin K10, are also reported to inactivate Akt [12-15].

The proteolytic activity of the ubiquitin-proteasome system (UPS) is important for many cellular processes [16]. The formation of polyubiquitin-protein conjugates, which are recognized and destroyed by the 26S proteasome, involves three components that participate in a cascade of ubiquitin-transfer reactions: ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2),

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and a specificity factor (E3) called the ubiquitin ligase. The E3 ligase controls the specificity of target-protein selection and the abundance of individual target proteins [16]. Although the molecular pathways and physiological roles of Akt ubiquitination have been recently studied [17-22], large aspects of Akt ubiquitination remain unclear. Here, we identify MULAN as a novel E3 ligase for Akt.

Results

Akt interacts with MULAN E3 ubiquitin ligase

To identify proteins that interact with Akt, we prepared a human full-length cDNA library derived from HeLa and liver Chang cells (Supplementary information, Data S1). The *in vitro*-transcribed and -translated protein pool from the cDNA library was screened for human Akt1-binding proteins with modified SMART technology. In this study, all experiments were performed using human Akt1, which is denoted 'Akt' unless otherwise specified. From this library, we isolated several positive cDNA clones, and among the isolates, one clone was identified as a major Akt-binding partner (data not shown). This clone contained several predicted functional domains: a transmembrane (TM) domain or a signal peptide at the N terminus, a second TM domain in the middle of the protein and a RING finger domain (i.e., the signature E3-ligase domain) at the C terminus. While our study has focused on the functional communications between this clone and Akt, Li *et al*. [23] named this protein as MULAN, which stands for mitochondrial ubiquitin ligase activator of NF-κB.

In vitro pulldown studies showed that Akt physically interacts with MULAN (Figure 1A). The intermolecular association between endogenous Akt and MULAN was investigated using a co-immunoprecipitation assay (Figure 1B). Human Akt has three isoforms: Akt1, Akt2, and Akt3. An Akt isoform-specific immunoprecipitation assay revealed that MULAN interacted with Akt1 and Akt2 but not with Akt3 (Figure 1C). In addition, MULAN depletion increased the protein levels of Akt1 and Akt2 but not Akt3 (Supplementary information, Figure S1). Akt2, but not Akt3, has been reported to translocate to the mitochondria [24]. The mitochondrial translocation of Akt1 is controversial [24-26]. However, our experimental system revealed that Akt1 could translocate to the mitochondria (Supplementary information, Figure S2A). Additionally, confocal microscopy revealed that Akt1 colocalized with MULAN (Supplementary information, Figure S2B). An *in vitro* binding assay using a series of Akt deletion mutants revealed that the kinase domain (KD) of Akt was primarily associated with MULAN (Figure 1D and 1E).

Akt ubiquitination and degradation are directly regulated by MULAN

To determine a functional role for the interaction between Akt and MULAN, we investigated whether MULAN functions as an E3 ligase for Akt. MULAN expression resulted in a decrease in Akt protein levels in an E3-ligase activity-dependent manner. In addition, the proteasome inhibitor MG132 completely reversed this decrease in cellular Akt protein levels (Figure 2A, lane 5). Next, *in vitro* and *in vivo* ubiquitination assays demonstrated that recombinant and endogenous Akt proteins were ubiquitinated in a MULAN E3-ligase activity-dependent manner (Figure 2B and 2C). The reverse trend was observed in MULAN siRNA-induced knockdown cells. MULAN siRNA transfection resulted in the inhibition of Akt ubiquitination in HEK293 cells (Figure 2D, left panel). Interestingly, serum/glucocorticoid-regulated kinase 1 (SGK1), which has high homology with Akt [27], was not affected by the depletion of endogenous MULAN (Figure 2D, right panel).

The ability to generate diverse substrate-ubiquitin structures is important for targeting proteins to different fates [28]. To address this, an *in vivo* ubiquitination assay was performed in HeLa cells expressing HA-tagged ubiquitin in which lysine 48 or 63 was mutated to arginine (HA-Ub WT, HA-Ub K48R, and HA-Ub K63R). As shown in Figure 2E, Ub K48R, but not Ub WT and Ub K63R, greatly reduced MULAN-mediated Akt ubiquitination, indicating that a K48-linked ubiquitination chain is formed during MULAN-mediated ubiquitination of Akt. These results indicate that MULAN E3 ligase specifically targets Akt, leading to its ubiquitination and subsequent proteasomal degradation.

pAkt is a preferential target for MULAN E3 ubiquitin ligase

As the upregulation of Akt kinase activity is strictly controlled by phosphorylation at serine 308 and threonine 473 [29], we examined whether the active/inactive status of Akt could affect Akt degradation by MULAN. To test this hypothesis, we first examined the interaction between endogenous MULAN and Akt upon stimulation with growth factor. Interestingly, the interaction between endogenous MULAN and Akt was detected in the presence of serum and insulin in HeLa cells (Figure 3A). Similarly, MULAN-induced Akt degradation preferentially occurred in serum-stimulated HEK293 cells (Figure 3B). In addition, *in vivo* ubiquitination assays demonstrated that serum stimulation induced endogenous Akt ubiquitination by MULAN (Figure 3C). Moreover, LY294002, a PI3K inhibitor that inhibits the

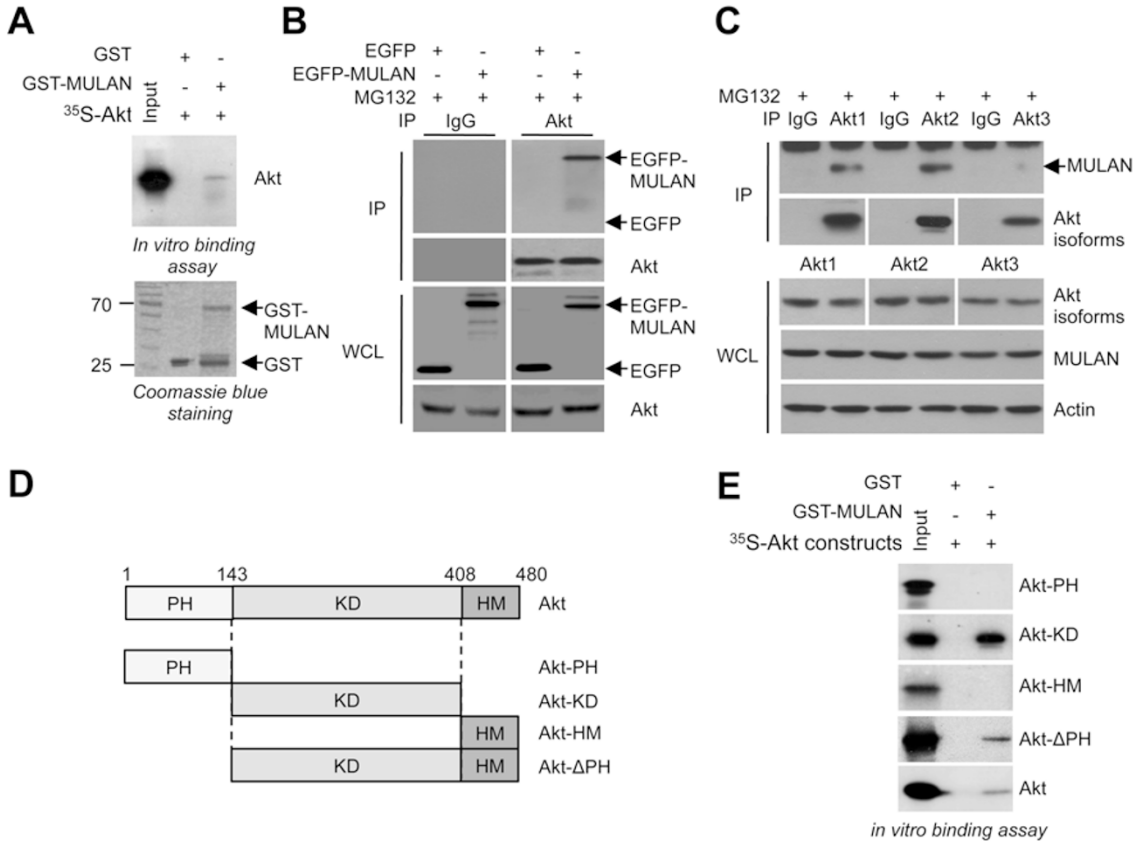

Figure 1 Akt interacts with the MULAN E3 ubiquitin ligase *in vitro* and *in vivo*. **(A)** *In vitro* interaction between Akt and MULAN. ^{35}S-methionine-labeled Akt was tested for an interaction with GST-tagged MULAN (GST-MULAN) using pull-down assays. **(B)** *In vivo* association between Akt and MULAN. After transfection with plasmids as indicated, HEK293 cells were treated with MG132 and then subjected to immunoprecipitation with an anti-Akt antibody and immunoblotting with anti-EGFP. Immunoglobulin G was used as a negative control. **(C)** Endogenous interaction between MULAN and Akt isoforms. MG132-pretreated HeLa cells were subjected to immunoprecipitation with Akt isoform-specific antibodies, and immunoblotting was performed using an anti-MULAN antibody. **(D)** The functional domain structure and map of the plasmids of the Akt deletion mutant. **(E)** The KD of Akt interacts with MULAN. The ^{35}S-methionine-labeled Akt deletion mutants were examined for interaction with GST-MULAN using pull-down assays.

phosphorylation of Akt, suppressed MULAN-induced Akt ubiquitination in serum-stimulated HEK293 cells (Figure 3C, lanes 5-8). These observations suggest a correlation between Akt activation status and MULAN-mediated degradation. To clarify this hypothesis, *in vitro* ubiquitination assays were performed in the presence of λ-PPase, which has activity towards phosphorylated serine, threonine, and tyrosine residues [30, 31]. Treatment with λ-PPase efficiently eliminated the phosphorylation of Akt residues and inhibited Akt ubiquitination (Figure 3D).

The aforementioned results led us to examine whether serum or growth factor could decrease the stability of endogenous Akt. Interestingly, we found that Akt protein levels were slightly decreased in serum-starved cells and substantially decreased in serum-stimulated cells, suggesting that phosphorylated Akt is more efficiently degraded than unphosphorylated Akt (Supplementary information, Figure S3). Additionally, we found that serum-induced Akt degradation was inhibited by MULAN depletion (Figure 3E) and that siRNA-induced MULAN depletion increased the protein levels of Akt and pAkt (Figure 6F). Furthermore, geldanamycin induced Akt degradation in both control and MULAN-depleted cells, suggesting that MULAN-induced Akt degradation was independent of Hsp90 inhibitor-induced Akt degradation (Supplementary information, Figure S4). Collectively, these results suggest that MULAN preferentially targets pAkt for ubiquitination.

Activated Akt is a polyubiquitination target of MULAN

Akt activation is induced by interdomain conforma-

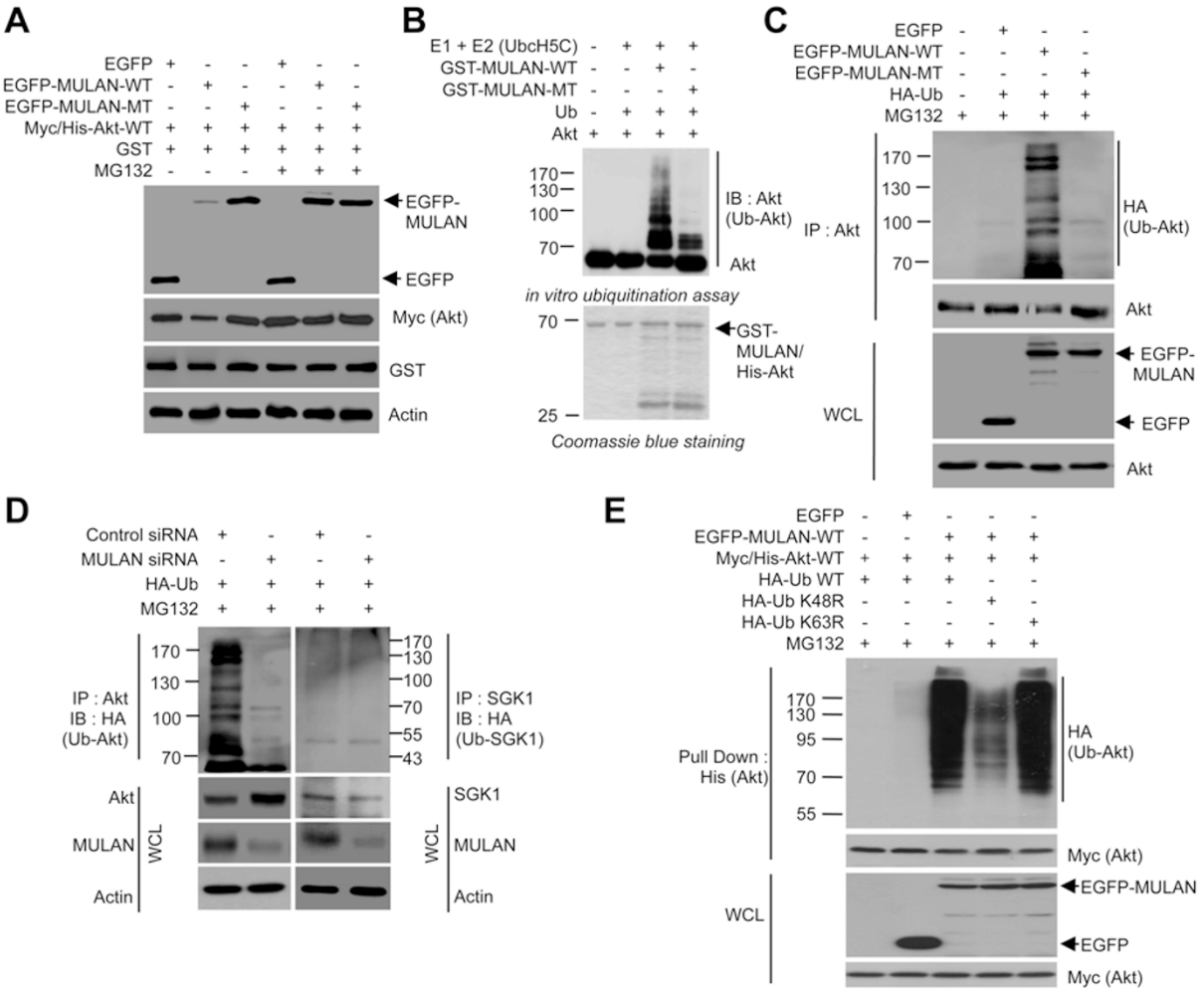

Figure 2 The ubiquitination and degradation of Akt are mediated by MULAN. **(A)** Cellular Akt protein levels were reduced by MULAN through the proteasomal degradation pathway in a RING-dependent manner. After transfection with plasmids as indicated, HEK293 cells were treated with MG132 and then subjected to immunoblotting with the indicated antibodies. The level of ectopic expression of GST was normalized to the transfection control. **(B)** Akt was ubiquitinated by MULAN *in vitro*. *In vitro* ubiquitination assays were performed as described in Materials and Methods. **(C)** Akt was ubiquitinated by MULAN *in vivo*. After transfection with plasmids as indicated, HEK293 cells were treated with MG132 and then subjected to *in vivo* ubiquitination assays as described in Materials and Methods. **(D)** The ubiquitination of Akt was ablated by MULAN siRNA transfection. After transfection with siRNAs and the HA-Ub plasmid as indicated, HEK293 cells were treated with 1 μM MG132 for 12 h and then subjected to *in vivo* ubiquitination assays. **(E)** K48-linked polyubiquitination was responsible for MULAN-dependent Akt ubiquitination. After transfection with plasmids as indicated into HeLa cells, an *in vivo* ubiquitination assay was performed.

tional change-mediated phosphorylation [32, 33]. We next examined the intracellular regulation of constitutively active Akt by MULAN in co-immunoprecipitation assays. Myristoylation signal-attached Akt (Akt-myr) is a constitutively active form of Akt [34]. Co-immunoprecipitation experiments revealed that MULAN interacts with ectopically expressed, functionally active Akt (Myc/His-Akt-WT and Myc/His-Akt-myr), but not dominant-negative Akt (Myc/His-Akt-DN), in HEK293 cells (Figure 4A). We confirmed that the ubiquitination of Akt is dependent on Akt activation in transfected HEK293 cells using *in vivo* ubiquitination experiments (Figure 4B). Similarly, LY294002 completely inhibited Akt phosphorylation and largely blocked MULAN-mediated Akt degradation (Figure 4C, lanes 4-6). Another PI3K/Akt inhibitor, wortmannin [35], also blocked MULAN-mediated Akt degradation (Supplementary information, Figure S5). These results suggest that MULAN preferentially binds to pAkt and promotes its degradation, consistent with our results that MULAN negatively regulates

active pAkt.

Lysine 284 in Akt is a major site of ubiquitination by MULAN

Akt is composed of an N-terminal pleckstrin homology (PH) domain, a central catalytic KD, and a short regulatory C-terminal hydrophobic motif (HM) [36]. The phosphorylation of Akt induces a conformational change in which the PH and HM domains become separated from the KD domain [37]. An *in vitro* ubiquitination

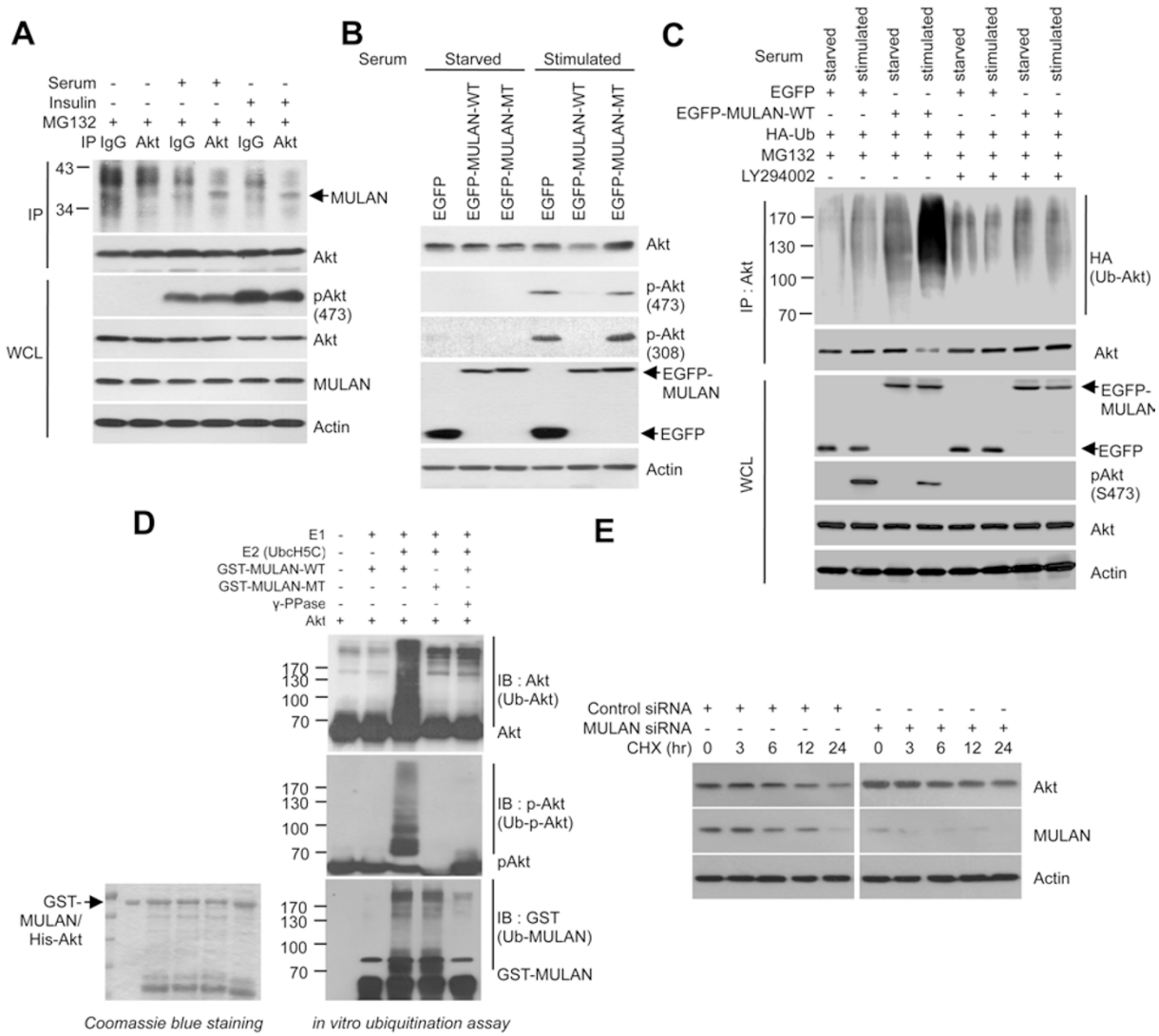

Figure 3 pAkt is a preferred substrate for ubiquitination by MULAN. **(A)** The interaction between MULAN and Akt was dependent on phosphorylated Akt. Serum-starved HeLa cells were treated with 10% serum or insulin along with MG132 for 6 h. An immunoprecipitation assay was then performed with an Akt antibody, followed by immunoblotting with an anti-MULAN antibody. (**B)** The cellular level of pAkt was decreased by MULAN. HeLa cells were transfected with plasmids as indicated. After 24 h of incubation, the medium was replaced with serum-free medium and the cells were subsequently stimulated with 10% serum for 2 h. Cells were lysed and immunoblotted with antibodies as indicated. **(C)** Cellular pAkt protein was ubiquitinated by MULAN. After transfection with plasmids as indicated, HeLa cells were incubated in a serum-free medium for 18 h. Cells were treated with 10 μM LY294002 and subsequently stimulated with 10% serum in the presence of 10 μM MG132. Cell lysates were immunoprecipitated and immunoblotted with the indicated antibodies. **(D)** Dephosphorylation of pAkt decreased the MULAN-mediated Akt ubiquitination *in vitro*. *In vitro* ubiquitination assays were performed with purified human Akt protein derived from Sf9 cells and λ-PPase. Ubiquitinated Akt and pAkt were detected by immunoblotting with the indicated antibodies. (**E**) MULAN siRNA treatment increased Akt protein stability. HeLa cells were transfected with control and MULAN siRNA and treated with 40 μg/ml cycloheximide for the indicated times, after which Akt protein turnover was investigated by immunoblotting.

assay demonstrated that MULAN specifically ubiquitinated Akt WT as well as Akt-KD (Figure 5A). We next determined which lysine residue in Akt-KD is required for protein stability and ubiquitination. To evaluate the correlation between Akt protein stability and mutations on lysine residues, we generated Akt lysine (K) to arginine (R) mutants of the putative ubiquitin-conjugation residues (K158, K163, K170, K183, K189, K214, K276, K284, K289, K297, and K307) in the KD domain by site-directed mutagenesis. As shown in Supplementary information, Figure S6, the degradation of Akt K284R was inhibited compared with that of Akt WT and the other Akt mutants. Furthermore, we verified that Akt K284R displayed significantly reduced ubiquitination via *in vivo* ubiquitination assays (Figure 5B). In addition, a cycloheximide-chase assay indicated that MULAN did not decrease the stability of Akt K284R compared with that of Akt WT (Figure 5C). The results suggest that lysine 284

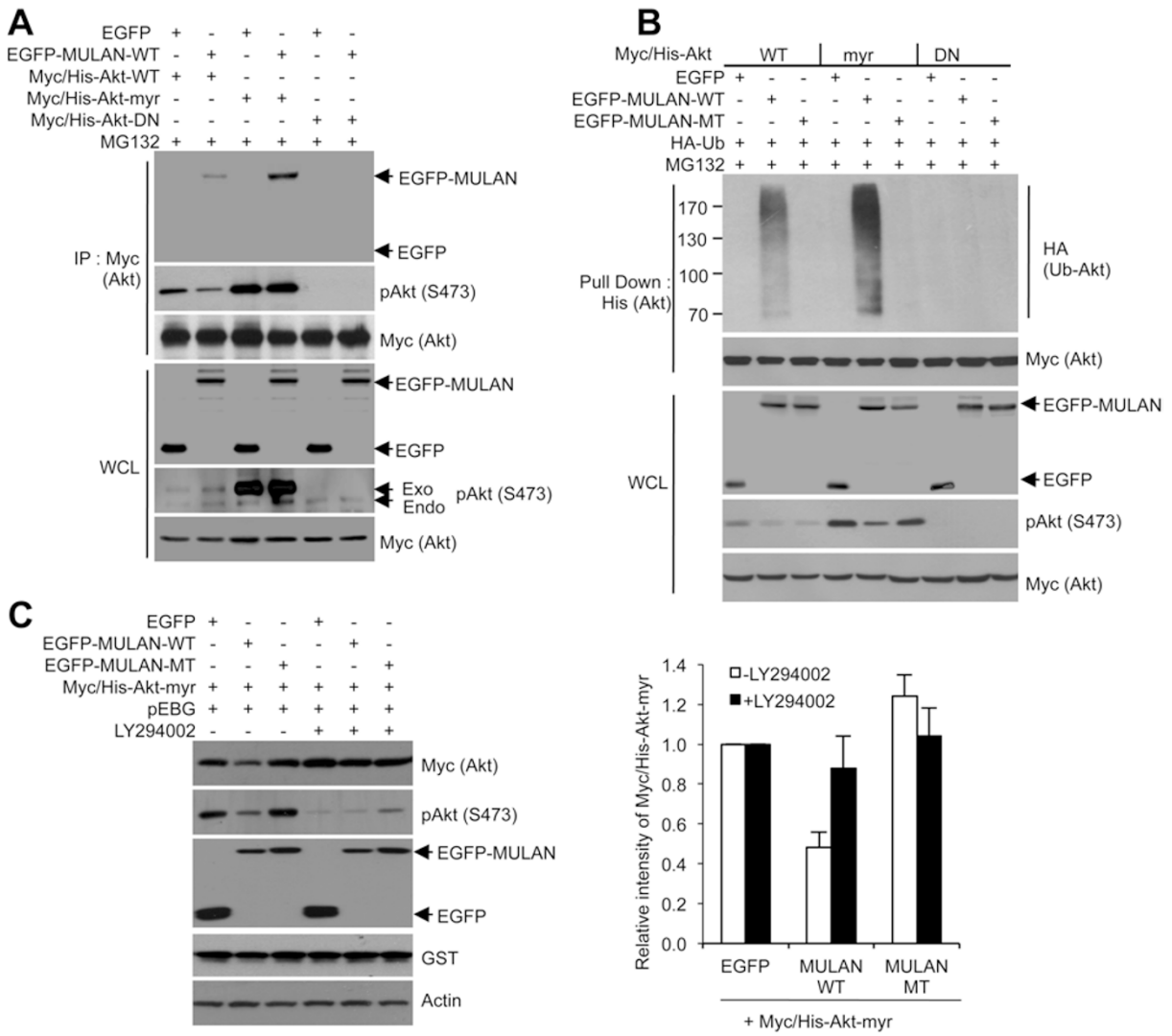

Figure 4 Activated Akt is a preferential target of the ubiquitin E3-ligase MULAN. **(A)** MULAN interacted with constitutively active Akt *in vivo*. After transfection with plasmids as indicated, HEK293 cells were treated with MG132 and then subjected to immunoprecipitation with an anti-myc antibody, followed by immunoblotting with the indicated antibodies. **(B)** Activated Akt was efficiently ubiquitinated by MULAN *in vivo*. After transfection with plasmids as indicated, HEK293 cells were treated with MG132 and then subjected to *in vivo* ubiquitination assays as described in Materials and Methods. **(C)** The MULAN-mediated proteolytic degradation of Akt was inhibited by LY294002. HEK293 cells were cotransfected with plasmids as indicated. After 24 h of incubation, cells were treated with 10 μM LY29402 for 4 h. Cells were lysed and immunoblotted with the indicated antibodies. The level of ectopic expression of GST was normalized to the transfection control. The graph represents the mean ± SD of the relative intensities of Akt from triplicate experiments.

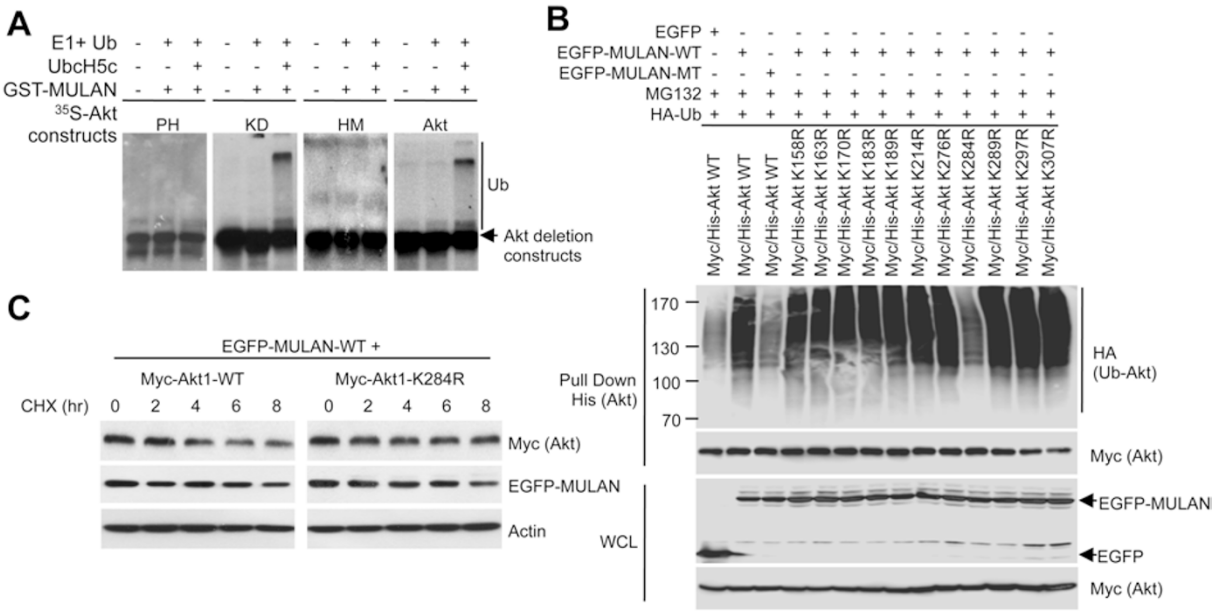

Figure 5 Lysine 284 of Akt is a site for MULAN-mediated polyubiquitination. **(A)** The KD of Akt is ubiquitinated by MULAN. A series of Akt deletion constructs were transcribed and translated in the presence of ^{35}S-methionine. The ^{35}S-labeled Akt mutants were subjected to ubiquitination by MULAN with *in vitro* ubiquitination assays. **(B)** Lysine 284 of Akt is a polyubiquitination site. HEK293 cells were cotransfected with plasmids as indicated. At 24 h after transfection, cells were treated with 10 μM MG132 and further subjected to *in vivo* ubiquitination assays as described in Materials and Methods. Whole-cell lysates were analyzed by immunoblotting with the indicated antibodies. **(C)** K284R-Akt is more stable than WT Akt. HeLa cells were cotransfected with the indicated plasmids and subsequently treated with 40 μg/ml cycloheximide. The cells were subjected to immunoblotting with the indicated antibodies.

in Akt is a specific residue for MULAN-mediated Akt ubiquitination.

The negative regulation of Akt signaling by MULAN

Akt plays an important role in regulating cell-cycle progression, cell survival, and cell growth [38]. We found that the growth of cells expressing enhanced green fluorescent protein EGFP-MULAN-WT was significantly suppressed compared with that of cells expressing EGFP-MULAN-MT or control EGFP (Figure 6A). MULAN-depleted cells grew faster than control cells (Supplementary information, Figure S7). MULAN inhibited the proliferation of HeLa cells in a concentration-dependent manner (Figure 6B). MTS assays also revealed that Akt-mediated increase in cell viability was reduced by MULAN overexpression in HeLa cells (Supplementary information, Figure S8). The cell growth inhibition caused by MULAN was verified by clonogenic and anchorage-independent soft agar assays (Figure 6C and 6D). HeLa cells expressing EGFP-MULAN-WT exhibited a 50% decrease in colony formation compared with cells expressing EGFP-MULAN-MT or control EGFP (Figure 6D). These results suggest that MULAN regulates the Akt downstream signaling pathway. GSK-3/β-catenin/TCF signaling is a critical downstream pathway of PI3K/Akt-mediated cell survival and proliferation [6, 39]. In Figure 6E, we show that the phosphorylation of GSK-3β and TSC2, both Akt downstream target proteins, was suppressed by MULAN in a RING-ligase activity-dependent manner. Additionally, the reverse trend was observed in a MULAN siRNA-dependent manner (Figure 6F). In the TCF/LEF1 response element-driven luciferase system, pTOPFLASH reporter activities were measured to confirm the inhibition of Akt downstream signaling by MULAN (Figure 6G). Taken together, our results indicate that MULAN suppresses cell growth by regulating the Akt signaling pathway. We then evaluated the effects of MULAN on Akt-induced cell migration using *in vitro* wound-healing assays. Ectopically expressed WT MULAN suppressed HeLa cell migration better than RING ligase MT MULAN (Figure 6H), and MULAN siRNA transfection increased HeLa cell migration (Supplementary information, Figure S9), indicating that the MULAN E3 ligase efficiently suppresses Akt and its downstream signaling.

Furthermore, we examined the effects of Akt K284R

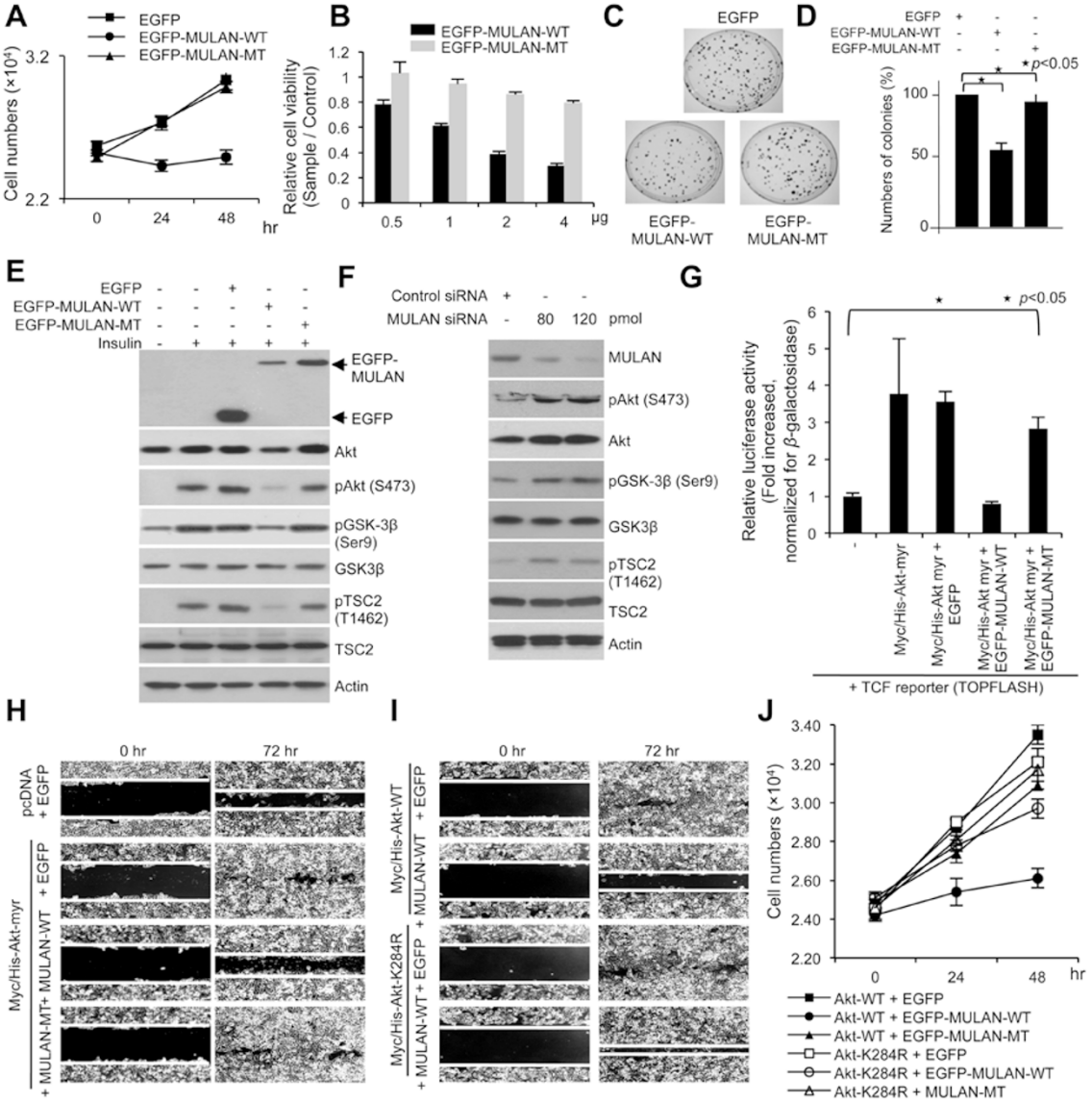

Figure 6 The cell growth function of Akt is suppressed by MULAN. **(A)** Cell growth is attenuated by MULAN. After transfection with plasmids as indicated in HeLa cells, the number of viable cells were counted using a hemocytometer every 24 h. The results are representative of three independent experiments. **(B)** MULAN inhibits cell viability in a dose-dependent manner. HeLa cells were transfected with plasmids as indicated. After 24 h of incubation, cell viability was determined by the MTS assay. **(C)** MULAN suppresses clonogenic growth. HeLa cells were transfected with plasmids as indicated, subsequently incubated in media containing G418 (500 μg/ml) for 2 weeks, and stained with crystal violet. **(D)** MULAN inhibits anchorage-independent growth. The soft-agar assay was performed with HeLa cells transfected with indicated plasmids. Colonies were counted 2 weeks after seeding. The results are representative of three independent experiments (mean ± SD are shown). The student's *t*-test was performed for statistical significance (**P* < 0.05). **(E-G)** Akt downstream signaling is regulated by MULAN. HeLa cells were transfected with the indicated MULAN plasmids **(E)** and MULAN siRNA **(F)**. After 24 h of incubation, the cells were lysed and immunoblotted with the indicated antibodies. The HeLa cells were cotransfected with TCF/LEF1 reporter plasmid, pTOPFLASH, and other plasmids as indicated. After 24 h of incubation, cells were lysed and the reporter luciferase activities driven by the TCF response element were determined **(G)**. **(H**, **I)** MULAN inhibited Akt-induced cell migration **(H)** but not K284R-Akt-induced cell migration **(I)**. HeLa cells were cotransfected with plasmids as indicated. After 48 h of transfection, 100% confluent cells were scratched to form a wound. The migration of the cells was visualized on a phase-contrast microscope at 72 h after inducing a wound. **(J)** The cell growth of K284R-Akt-expressing cells is not inhibited by MULAN. After transfection of HeLa cells with plasmids as indicated, the number of viable cells was counted using a hemocytometer every 24 h.

on cell growth and cell migration. MULAN suppressed Akt-induced cell growth more efficiently than Akt-K284R-induced cell growth (Figure 6J). Similarly, Akt-K284R-induced cell migration was not significantly inhibited by MULAN, in contrast to Akt WT-induced cell migration (Figure 6I). These results indicate that the MULAN E3 ligase efficiently suppresses Akt function by ubiquitination of Akt at lysine 284.

Akt is known to activate NF-κB, and MULAN has been reported to be an NF-κB activator [23, 45]. Therefore, it appears unclear whether MULAN induces NF-κB activity or inhibits Akt to suppress NF-κB activity. To clarify this issue, we examined NF-κB activity using a reporter plasmid containing NF-κB-binding sites (NF-κB luciferase reporter). HeLa cells were transfected with EGFP control, EGFP-MULAN-WT, and EGFP-MULAN-MT or cotransfected with plasmids for Myc/His-Akt WT. In these transfected HeLa cells, MULAN WT and MULAN MT increased NF-κB activity (up to 3-fold), however, the upregulation of NF-κB activity by Akt WT (up to 7-fold) was decreased in a MULAN E3 ligase-dependent manner (Supplementary information, Figure S10). This result indicates that although MULAN can upregulate NF-κB activity, this effect is independent of the Akt-mediated NF-κB pathway.

Discussion

The present study examined the role of MULAN in the negative regulation of Akt signaling. MULAN specifically interacted with Akt *in vivo* and *in vitro* through the KD of Akt. Although this domain is highly conserved (90%-95%) among the three Akt isoforms, MULAN interacts with Akt1 and Akt2 but not with Akt3. However, SGK1, another closely related serine/threonine kinase, was not bound or ubiquitinated by MULAN (data not shown and Figure 2D). Additionally, MULAN was not phosphorylated by Akt kinase in an *in vitro* kinase assay (data not shown).

We found that the interplay between Akt and MULAN depends on the phosphorylation status of Akt and tightly regulates Akt protein stability. Serum stimulation, which induces Akt phosphorylation, enhanced the interaction between Akt and MULAN, as well as Akt ubiquitination and degradation. Akt phosphorylation-dependent proteolytic degradation was also investigated using the ectopic expression of a constitutively active Akt (Myc/His-Akt-myr). Co-immunoprecipitation assays revealed that MULAN bound to phosphorylated active Akt-myr but not to unphosphorylated inactive Akt-DN *in vivo*. Furthermore, treatment with LY294002 and wortmannin inhibited the MULAN-mediated proteolytic degradation of Myc/His-Akt-myr. Conversely, MULAN depletion increased Akt protein stability and pAkt levels. Collectively, our data indicate that MULAN preferentially binds to pAkt and promotes its degradation.

Structurally, Akt becomes fully activated through the phosphorylation of a specific threonine residue (T308) within the T loop of the catalytic domain and a specific serine residue (S473) located in a C-terminal, noncatalytic region [40]. The inactive state of Akt is maintained through an interaction between its PH and KDs that prevents the activation loop from being phosphorylated by its upstream kinase phosphoinositide-dependent protein kinase-1 [37, 41]. Although the precise structural mechanism remains unknown, our results support the conclusion that the phosphorylation of Akt exposes the preferential binding site for MULAN in the Akt KD. First, the pull-down assay data indicated an interaction between MULAN and the KD of Akt. Second, competition-based peptide-binding assays with synthesized short phosphopeptide motifs of Akt revealed that the binding affinity of MULAN for the phosphopeptides was similar to that of the control nonphosphopeptides of Akt (data not shown). Third, a phosphomimetic Akt (T308D/S473D, constitutively active form) interacted with MULAN (data not shown). These results also suggest that phosphorylated residues in Akt are not critical for its interaction with MULAN.

Protein degradation by UPS is used to control many cellular processes [16]. Several reports have described UPS-mediated Akt proteolytic degradation in different types of cells [42]. Akt is stabilized in the presence of Hsp90, and Hsp90 inhibitors induce Akt degradation [18]. However, we found that MULAN-induced Akt degradation was independent of Hsp90 inhibitor-induced Akt degradation. Riesterer *et al.* [42] reported that the inhibition of vascular endothelial growth factor (VEGF) signaling by the VEGF inhibitor PTK787/Zk222584 results in the specific degradation of cellular Akt by UPS. In addition, rapamycin, which inhibits the mammalian target of rapamycin, also abrogates the VEGF-protective effect on UPS-mediated Akt degradation. Yan *et al*. [43] reported that dendritic Akt is selectively degraded by UPS in primary hippocampal neurons. They indicated that the instability of Akt in dendrites is required for the formation of neuronal polarity. Despite the observation that UPS-mediated Akt degradation occurs under different cellular conditions, it remains unclear which specific E3 ligases are involved in Akt degradation by UPS. A recent report demonstrated that the tetratricopeptide repeat domain 3 is an Akt-specific E3 ligase that binds to pAkt and facilitates its ubiquitination and degradation in the nucleus [20]. Although activated Akt can be localized

to the plasma membrane as well as to the cytoplasm and nucleus [44], our study implies that MULAN negatively regulates cytosolic pAkt because MULAN is predominantly localized in the mitochondria [23].

A previous study reported the cellular localization of MULAN in mitochondria and MULAN-mediated regulation of the apoptotic signaling pathway [45]. Additionally, other groups reported the caspase-dependent cleavage of Akt during apoptosis [46]. Although we cannot disprove the possibility that caspase activation by MULAN influences Akt signaling, MULAN-mediated Akt degradation is irrelevant to the caspase signaling pathway, as ectopically expressed Akt was still degraded by EGFP-MULAN-WT in HeLa cells in the presence of the caspase inhibitor Z-VAD (Supplementary information, Figure S11).

To date, the residues in Akt that are responsible for the polyubiquitin-dependent protein degradation have not been reported. Although regions of Akt-KD interacted with MULAN, we were not able to exclude the possibility that Akt-PH and Akt-HM might have lysine residues that can be ubiquitinated. As demonstrated in Figure 5A, the KD of Akt was sufficient for ubiquitination by MULAN. To identify the site in Akt that is ubiquitinated by MULAN, we introduced point mutations of 11 putative lysine residues in the Akt KD. Our data demonstrated that a single point mutation at K284 in Akt is sufficient to diminish Akt ubiquitination. However, in our experiments, we could not exclude the possibility that simultaneous ubiquitination at multiple sites affects the stability of Akt.

Akt plays a critical role in promoting a variety of cellular processes that are involved in cell proliferation and migration through the phosphorylation of downstream target substrates [36]. We demonstrated that the overexpression of MULAN decreased cell growth and cell proliferation in an E3-ligase activity-dependent manner. GSK-3β and TSC2 are well-known targets of Akt that control protein synthesis, cell proliferation, differentiation, motility, and apoptosis through their proapoptotic activities [47, 48]. Several reports suggest that the proapoptotic activity of GSK-3β is inhibited by Akt through the phosphorylation of serine 9 in GSK-3β [6]. The phosphorylation of threonine 1 462 in TSC2 is directly induced by Akt activation in the mediation of growth signals for the insulin-signaling pathway [49]. Our present study demonstrates that MULAN inhibits the phosphorylation of GSK-3β and TSC2 in a RING-ligase activity-dependent manner. The MULAN-mediated inhibition of Akt signaling revealed by the pTOPFLASH reporter and wound-healing assays indicates that MULAN controls these cellular processes through the regulation of Akt signaling. Moreover, we found that MULAN-induced NF-κB activation was independent of the E3-ligase activity of MULAN and Akt-induced NF-κB activation was decreased by MULAN in an E3-ligase activity-dependent manner. Recently, Zhang *et al*. [45] reported that MULAN/GIDE and RING-finger domain deleted-MULAN/GIDE weakly activated NF-κB, however, the overexpression of IKKβ-KA and IκBα (SS/AA), which inhibits MULAN/GIDE-induced NF-κB, did not inhibit the MULAN/GIDE-induced anti-cell survival effect. The mechanisms of MULAN-induced NF-κB activation remain unknown, and NF-κB activation is independent of MULAN anti-cell survival functions. Therefore, although MULAN can upregulate NF-κB activity, this effect would be independent of the Akt-mediated NF-κB pathway.

The results of this study suggest that MULAN negatively regulates Akt signaling, facilitating the control of multiple cellular processes. Our results also provide a possible future target for Akt regulators, although the clinical significance of MULAN warrants further investigation.

Materials and Methods

Plasmids

The coding regions of Akt and MULAN were generated by PCR from cDNA synthesized from HeLa cells. Human Akt1 cDNA was cloned into pcDNA3.1-Myc/His (Invitrogen, Carlsbad, CA, USA), pGEX6p (Promega, Madison, WI, USA) and pET28 (Novagen, EMD Chemicals Inc, Merck, Darmstadt, Germany) vectors. MULAN cDNA was cloned into pEGFP-C (Clontech, Mountain View, CA, USA) and pGEX6p vectors. The fragments of Akt were cloned into the pcDNA3.1 vector (Invitrogen). The dominant-negative Akt (DN-Akt: T308A and S473A), Akt lysine mutants, and RING domain-mutant MULAN (MULAN MT: C302S and C305S) were created using the QuikChange site-directed mutagenesis kit (Stratagene, Santa Clara, CA, USA) according to the manufacturer's instructions. The HA-ubiquitin (HA-Ub) plasmid pMT123 was kindly provided by Dr Dirk Bohmann (University of Rochester, Rochester, NY, USA). HA-Ub mutant constructs (HA-Ub-K48R and HA-Ub-K63R) were kind gifts from Dr Zhijian Chen (University of Texas Southwestern Medical Center, USA). The sequences of primers used for PCR are listed in Supplementary information, Table S1.

Recombinant proteins

GST and GST-MULAN were expressed in *Escherichia coli* BL21 at 37 °C with 0.1 mM IPTG for 1 h. The cells were collected and resuspended in NP-40 lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 1 mM DTT, 1 mM EDTA, lysozyme, and protease inhibitors). The recombinant proteins were isolated and eluted using glutathione-Sepharose 4B beads according to the manufacturer's instructions. Each eluate was dialyzed in a Slide-A-Lyzer dialysis cassette (Pierce, Rockford, IL, USA) and concentrated using an Amicon Ultra-15 device (Millipore, Billerica, MA,

USA). His-Akt was purchased from Millipore.

Antibodies and reagents

The following primary antibodies were used: EGFP, myc-tag, phosphorylated Akt (pAkt) (S308), pAkt (T473), Akt, pGSK-3β, TSC2, pTSC2 (T1462) (all from Cell Signaling Technology, Danvers, MA, USA), HA-tag, GSK-3β (both from Santa Cruz Biotechnology, Santa Cruz, CA, USA) and SGK1 (Millipore). The MULAN polyclonal antibody was generated by immunizing rabbits with two synthetic peptides comprised of amino-acids N-SGERPKGIQETEEM-C and N-SRAKPEDRESLKSAC-C (Abfrontier, Seoul, Korea). Transient transfections were performed using the Lipofectamine 2000 reagent (Invitrogen). Z-VAD and MG132 were obtained from Sigma-Aldrich (St Louis, MO, USA). LY294002 and wortmannin were purchased from Cell Signaling Technology. The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay kit was purchased from Promega.

In vitro binding assay

The ^{35}S-labeled protein and protein pools obtained from *in vitro* transcription/translation reactions were incubated with 3 μg of glutathione-Sepharose 4B beads (GE Healthcare, Uppsala, Sweden) bound to GST or GST-Akt recombinant proteins for 3 h at 37 °C in 0.5% NP-40 buffer, washed three times, and boiled in SDS sample buffer. Bound proteins were separated by SDS-PAGE and visualized using autoradiography.

Immunoprecipitation

For immunoprecipitation assays, cell lysates in CHAPS buffer (0.5% CHAPS, 10 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 1 mM EGTA, 5 mM β-mercaptoethanol, and 10% glycerol) were incubated with the indicated antibody under constant rotation overnight at 4 °C. Protein A/G PLUS-Agarose beads (Santa Cruz Biotechnology) were added and the slurry was incubated for an additional 2 h followed by extensive washing.

In vitro ubiquitination assay

Purified His-Akt from insect cells (Upstate, Millipore) or purified λ-protein phosphatase (λ-PPase, New England Biolabs, Ipswich, MA, USA)-treated His-Akt was incubated with 150 ng recombinant E1 (Boston Biochem, Cambridge, MA, USA), 250 ng recombinant UbcH5C (Boston Biochem), 500 ng GST-MULAN and 5 μg His-ubiquitin (Boston Biochem) in ubiquitination buffer (50 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 15 μM $ZnCl_2$, 0.3 mM DTT, 0.006% DTT, 2 mM ATP, 10 U creatine phosphokinase, and 10 mM phosphocreatine) for 90 min at 30 °C. For λ-PPase-treated ubiquitination reactions, His-Akt with or without λ-PPase was incubated in ubiquitination buffer for 1 h at 30 °C, after which 2× phosphatase inhibitor cocktail was added (Roche Applied Science), and the reaction mixture was incubated with E1, E2, GST-MULAN protein, and ubiquitin. The reaction products were resolved by 8% SDS-PAGE followed by immunoblotting with anti-Akt antibodies.

In vivo ubiquitination assay

Transfected and MG132-treated cells were washed with PBS and then lysed in 200 μl of denaturing lysis buffer (50 mM Tris-HCl, pH 7.4, 0.5% SDS, and 70 mM β-mercaptoethanol) by vortexing and boiling for 15 min at 95 °C. The lysates were diluted with 800 μl of CHAPS buffer containing protease inhibitor cocktail and MG132. The lysates were immunoprecipitated with anti-Akt antibody and protein agarose A/G PLUS-Agarose beads. The beads were washed five times in CHAPS lysis buffer and then boiled. Ubiquitinated Akt was visualized by immunoblotting with anti-HA antibody. For His pull-down assays, the denatured lysates were diluted with 800 μl of buffer A (50 mM NaH_2PO_4, 300 mM NaCl, and 10 mM imidazole, pH 8.0) containing protease inhibitor cocktail and MG132. The diluted lysates were incubated with Ni-NTA beads overnight at 4 °C. The beads were washed five times with buffer B (50 mM NaH_2PO_4, 300 mM NaCl, and 20 mM imidazole, pH 8.0). The bound proteins were eluted by boiling in SDS-PAGE sample buffer. The eluted proteins were immunoblotted with anti-HA antibody.

RNA interference

siRNAs for MULAN were purchased from Ambion (Austin, TX, USA). MULAN siRNA (100 pmol) was transfected into HEK293 cells (5×10^5 cells) with Lipofectamine RNAiMAX (Invitrogen) for 5 h in serum-free medium. After changing the medium, the cells were incubated for 48 h and harvested.

Cell viability assay

Cell viability was assessed using the MTS assay. The results are graphically represented as the measured cell viability ratio normalized to the control.

Clonogenic survival assay

HeLa cells (4×10^4) were transfected with EGFP control plasmid, EGFP-tagged MULAN WT, or MULAN MT. After 2 weeks, the colonies were fixed and stained with 0.1% crystal violet. For the anchorage-independent soft-agar assay, HeLa cells (2×10^4) transfected with the above plasmids were added to 3 ml of DMEM with 0.45% low-melting agarose and 10% FBS and overlaid onto a 2-ml/six-well dish of DMEM with 0.9% agarose and 10% FBS. The colonies were counted after 2 weeks.

Luciferase reporter gene assay

The TCF-responsive luciferase construct pTOPFLASH (Upstate) was used to study transcriptional activity. The pTOPFLASH vector has six TCF-binding sites, three in each direction. The relative luciferase activity was determined after a 48-h transfection, normalized to β-galactosidase activity, and presented as fold activation with SD. The results are the averages of three independent experiments.

Statistical analysis

Statistical analysis was performed using the χ^2 test or Fisher's exact test and Spearman's rank correlation coefficient analysis. $P < 0.05$ was considered significant.

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(**Supplementary information** is linked to the online version of the paper on the *Cell Research* website)