Akt SUMOylation Regulates Cell Proliferation and Tumorigenesis

Rong Li, Jie Wei, Cong Jiang, Dongmei Liu, Lu Deng, Kai Zhang, and Ping Wang

Abstract

Proto-oncogene Akt plays essential roles in cell proliferation and tumorigenesis. Full activation of Akt is regulated by phosphorylation, ubiquitination, and acetylation. Here we report that SUMOylation of Akt is a novel mechanism for its activation. Systematically analyzing the role of lysine residues in Akt activation revealed that K276, which is located in the SUMOylation consensus motif, is essential for Akt activation. Ectopic or endogenous Akt1 could be modified by SUMOylation. RNA interference–mediated silencing of UBC9 reduced Akt SUMOylation, which was promoted by SUMO E3 ligase PIAS1 and reversed by the SUMO-specific protease SENP1. Although multiple sites on Akt could be SUMOylated, K276 was identified as a major SUMO acceptor site. K276R or E278A mutation reduced SUMOylation of Akt but had little effect on its ubiquitination. Strikingly, these mutations also completely abolished Akt kinase activity. In support of these results, we found that expression of PIAS1 and SUMO1 increased Akt activity, whereas expression of SENP1 reduced Akt1 activity. Interestingly, the cancer-derived mutant E17K in Akt1 that occurs in various cancers was more efficiently SUMOylated than wild-type Akt. Moreover, SUMOylation loss dramatically reduced Akt1 E17K–mediated cell proliferation, cell migration, and tumorigenesis. Collectively, our findings establish that Akt SUMOylation provides a novel regulatory mechanism for activating Akt function. Cancer Res; 73(18); 5742–53. ©2013 AACR.

Introduction

Posttranslational modification (PTM) is essential for the proper functions of numerous eukaryotic proteins. Lysine residue serves as the key acceptor site for PTMs including ubiquitination, acetylation, SUMOylation, and methylation (1–3). Among the various lysine modifications, SUMOylation has emerged as an important PTM that plays essential roles in various biologic functions including cell growth, migration, and tumorigenesis (4–6). SUMOylation is an enzymatic cascade reaction catalyzed by covalently conjugating small ubiquitin-related modifiers (SUMO) to an internal lysine residue in target proteins via its carboxyl-terminal glycine in the processed SUMO (4). Attachment of SUMO to a protein may affect the protein activity, subcellular localization, and stability (4). Accumulating evidence indicates that SUMOylation can target a wide variety of proteins including nuclear transcription factors, membrane, and cytosolic proteins (4, 7). Compared with a large amount of nuclear transcription factor, only a few protein kinases, such as Aurora-B kinase, MEK, FAK, and ERK5, have been identified as SUMO targets (8–11). Moreover, regulation of various protein kinases by SUMOylation and the related biologic functions remains unclear.

Akt is a central serine/threonine protein kinase involved in numerous essential biologic processes including cell proliferation, apoptosis, cell migration, metabolism, and tumorigenesis (12, 13). Akt contains a PH domain at the N terminus and a central kinase domain followed by a C terminal tail (12, 13). The activation of Akt is precisely controlled under physiologic conditions (13). Deregulated activation of Akt is related to many diseases such as cancer and metabolic diseases (13). Akt is activated by extracellular signals via recruitment to plasma membrane through binding of its PH domains to PI(3,4,5)P3, which results in phosphorylation at T308 and S473 (13, 14). Recent evidence indicates that lysine modification is critical for Akt phosphorylation, subcellular localization, stability, and activity (14). For example, TRAF6 or SKP2-mediated K63 polyubiquitination at lysine residues in PH domain has been shown as an essential regulatory mechanism for Akt activation (15, 16). Ubiquitination of Akt by CHIP, Nedd4, TTC3, and MULAN regulates Akt stability (17–19). Deacetylation of Akt at lysines in PH domain promotes its membrane translocation and activation (20). Protein sequence analysis reveals that Akt contains 34 lysine residues. However, modification of most lysine residues remains largely unknown.
SUMOylation of Akt

Materials and Methods

Cell culture and transfection

Cell lines were obtained from American Type Culture Collection or the Chinese Academy of Sciences Committee Type Culture Collection Cell Bank and authenticated by the cell banks with short tandem repeat analysis. Mouse embryonic fibroblast (MEF) cells were provided by Dr. Xin Ge (Yale University, New Haven, CT). HEK293T, A549, and MEF cells were cultured in Dulbecco’s Modified Eagle’s Media (DMEM) supplemented with 10% heat-inactivated FBS (Invitrogen). H1975 and H1299 cells were cultured in RPMI-1640 supplemented with 10% heat-inactivated FBS. Transfections were conducted using the calcium phosphate–DNA coprecipitation method for HEK293T cells and Lipofectamine 2000 for H1975 and H1299 cells following the manufacturer’s manual.

Antibodies and plasmids

Antibodies against hemagglutinin (HA) and GFP were obtained from Santa Cruz: Flag was from Sigma; Akt1, phospho-Akt, and phospho-Akt-substrate were from CST; UBC9, PIAS1, phospho-GSK3β, and GSK3α/β were from Epitomics and SENP1 was from Bethyl. Expression plasmids of Akt and its mutants were cloned into pcDNA3.1 vector with Flag, HA, or GFP tag at the N terminus or C terminus or pcDNA3 using standard cloning method. HeLa cells were transfected with the indicated constructs, cultured for 24 hours, and visualized after fixation using a confocal microscope (Leica).

siRNA knockdown

H1975 or H1299 cells were transfected with control or siPIAS1 (5′-GGU CCA GUU AAG GUU UUG UTT-3′) or siUBC9 (5′-GGG AUU GGU UUG GCA AGA A dTdT-3′) using Lipofectamine 2000. Cells were harvested after transfection for 48 to 72 hours and analyzed using Western blotting.

In vitro wound-healing assay

Confluent H1299 cells grown in 24-well plates were treated with MMC (10 μg/mL) for 6 hours to inactivate cell proliferation. The cells were wounded and images were captured after 12 hours as described previously (25). Three independent experiments were carried out.

Proliferation assay

For MTT assay, H1299 cells were seeded in a 96-well plate. Cells were harvested every 24 hours, the MTT solution was added for 4 hours. The reactions were stopped by addition of dimethyl sulfoxide (DMSO) solution for 20 minutes, and the samples were measured at 490 nm. Three independent experiments were carried out. 5-Ethynyl-2′-deoxyuridine (EdU) labeling was conducted using the EdU Apollo567 DNA Kit (Cat. C10031 Guangzhou Ribobio) according to the instruction.

A549-derived xenograft in NOD/SCID mice

For the xenograft formation, 6 × 10⁶ A549 cells were harvested and injected subcutaneously into nude mice. Five-week-old male BALB/c/a nude mice (National Rodent Laboratory Animal Resources, Shanghai, China) weighing about 20 g each were randomly divided into each group of 5. The body weight of each mouse was recorded every 7 days. At the same time, solid tumor volume was determined using Vernier caliper measurements and the formula $A \times B^2 \times 0.52$, where $A$ is the longest diameter of the tumor and $B$ is the shortest diameter of the tumor. Approximately 4 weeks later, tumors were harvested and imaged.

Immunoprecipitation and endogenous SUMOylation of Akt

To detect the SUMOylation of endogenous Akt, H1975 cells were lysed in the presence of 50 m mol/L N-ethylmaleimide (NEM). Endogenous Akt was immunoprecipitated using an Akt antibody and subjected to SDS-PAGE and Western blotting. The SUMOylation of endogenous Akt was examined using an anti-Akt or anti-SUMO antibody.

Subcellular fractionation

The subcellular fractionation was conducted as described previously (24). The fractions were subjected to an in vivo SUMOylation assay and Western blotting.

BiFC and immunofluorescence assays

For the BiFC assay, YN (YFP N terminus 1–154)–SUMO1 and YC (YFP C terminus 155–238)–Akt were cloned into pcDNA3 using standard cloning method. Hela cells were transfected with the indicated constructs, cultured for 24 hours, and visualized after fixation using a confocal microscope (Leica).

In vitro phosphorylation assay

GST-GSK3β protein was purified from Escherichia coli by standard protocols. Flag-Akt1 or mutant forms were expressed in HEK293T cells and purified using M2 beads (Sigma) for immunoprecipitation. Kinase assays were conducted as described in our previous study (21).

Ubiquitination and SUMOylation assay

In vitro ubiquitination assay was described in our previous study (22). SUMOylation assay was conducted as described previously (23). Briefly, transfected cells were lysed using denaturing Buffer A (6 mol/L guanidine-HCl, 0.1 mol/L Na₂HPO₄/NaH₂PO₄ 10 m mol/L imidazole, pH 8.0) and SUMOylated proteins were purified using Ni-NTA beads. The beads were then washed sequentially with Buffer A, Buffer B (8 mol/L urea, 0.1 mol/L Na₂HPO₄/NaH₂PO₄, pH 8.0, 0.01 mol/L Tris-HCl, pH 8.0, 10 m mol/L β-mercaptoethanol), Buffer C (8 mol/L urea, 0.1 mol/L Na₂HPO₄/NaH₂PO₄, pH 6.3, 0.01 mol/L Tris-HCl, pH 6.3, 10 m mol/L β-mercaptoethanol) with 0.2% Triton X-100, and Buffer C. Washed beads were incubated with 40 μL elution buffer (200 m mol/L imidazole, 0.15 mol/L Tris-HCl, pH 6.7, 30% glycerol, 5% SDS, 0.72 mol/L β-mercaptoethanol) at room temperature for 30 minutes. The input and elutes were analyzed using Western blotting.

Immunooprecipitation and Western blotting

Immunoprecipitation and Western blotting have been described previously (21). Proteins were resolved by SDS-PAGE and immunoblots were analyzed using the Odyssey System.

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Statistical analysis
The significance of differences was determined using the Student t test. All quantitative data are expressed as means ± S.D. P < 0.05 was regarded as significant difference.

Results
Systemically analyzing the role of lysine residues in Akt phosphorylation and activation
Given that lysine modifications are often essential for protein functions, we were interested in investigating the role of lysine in Akt phosphorylation and activity. Mouse Akt contains 34 lysine residues distributing in all the domains including PH, kinase, and C terminal domains. We replaced each individual lysine residue of mouse Akt with arginine and their phosphorylation and activity were compared with wild-type Akt in HEK293T cells using an in vitro kinase assay (Fig. 1A). Among these mutants, K14R greatly impaired the phosphorylation of S473 and T308 as well as kinase activity. This is consistent with recent report that K63 ubiquitination of this residue is essential for Akt kinase activity (15). Moreover, K183R reduced the phosphorylation at S473 and kinase activity but no measurable effect on T308 phosphorylation. We also noticed that K168, K179, and K276R lost the kinase activity but had minor effect on its phosphorylation at T308 and S473. Consistent with previous report, K179 has been shown to be essential for Akt kinase activity (26). To our knowledge, the effect of K168R, K183R, and K276R on Akt kinase activity and phosphorylation has not been reported. The essential roles of these sites on Akt kinase activity were confirmed by coexpression of these mutants with GSK3 in cells (Fig. 1B).

Figure 1. Lysine residues involved in Akt activity. A, in vitro kinase assay measuring Akt activity. Flag-tagged wild-type (WT) or Akt mutants were expressed in HEK293T cells and purified using anti-Flag antibody. The activity was measured using an in vitro kinase assay. B, cells were transfected as indicated. The phosphorylated GSK3β and Akt were examined using Western blotting.
**SUMOylation of Akt in vitro and in vivo**

To understand how K168R, K183R, and K276R mutation affects Akt activity, we analyzed the amino acid sequence surrounding them. Interestingly, we found that K276 is surrounded by a typical SUMOylation consensus motif (Supplementary Fig. S1A), which is ψKXE/D or E/DXXψ, where ψ represents a hydrophobic amino acid (such as isoleucine or valine), K is the acceptor lysine residue to which SUMO is covalently attached, and X represents any amino acid (4). Thus, we examined whether Akt is modified by SUMOylation. To this end, we expressed Flag-Akt with wild-type His-SUMO1 or SUMO1 (ΔGG) mutant without the C terminal diGlycine motif, which is required for its conjugation to substrates. Then, the SUMOylated proteins were purified using Ni²⁺-NTA resin, the SUMOylated Akt1 was clearly detected with multiple slower migrating bands with molecular weight bigger than 70 kDa (the normal Flag-Akt is around 60 kDa) in SDS-PAGE gel. In contrast, SUMO1 (ΔGG) mutant completely lost the ability to promote Akt SUMOylation (Fig. 2A). Similar results were obtained from an immunoprecipitation-based SUMOylation assay (Supplementary Fig. S1B). These results indicate that band shift of Akt was indeed due to the covalent attachment of SUMO. Moreover, we could detect the SUMOylation of endogenous Akt (Fig. 2B and Supplementary Fig. S1C), although the signal is weak. This is consistent with the notion that usually less than 1% amount of the target proteins was SUMOylated at steady state (5). Stimulation of cells with Insulin for 10 minutes markedly increased the levels of SUMOylated Akt (Fig. 2B). The SUMOylation of Akt1 was also confirmed using an E.coli–based in vitro SUMOylation assay (Supplementary Fig. S1D). Three functional SUMO proteins, SUMO1, 2, and 3, have been identified in human (27). Thus, we examined SUMOylation of Akt by SUMO2 and 3. Interestingly, our data showed that multiple SUMOylated Akt1 bands were detected in all 3 SUMO-purified complexes (Supplementary Fig. S1E). Akt2, but not Akt3, showed SUMOylation in cells as well (Supplementary Fig. S1F). Thus, our data showed that Akt is a target of SUMOylation.

SUMOylation is an enzymatic cascade reaction catalyzed by E1-activating enzyme containing a SUMO activating enzyme subunit (SAE1)–SAE2 heterodimer, the E2 conjugating enzyme UBC9, and E3 ligases (5). Currently, UBC9 is the only E2 identified in mammalian cells (4, 5). Fusion with UBC9 has been shown to be able to significantly promote the SUMOylation of targets (28, 29). As expected, fusion of wild-type but not inactive UBC9 to the COOH terminus dramatically increased the SUMOylation of Akt1 (Supplementary Fig. S1G). Moreover, depletion of endogenous UBC9 by siRNA markedly reduced the Akt SUMOylation (Fig. 2C). These results indicate that SUMOylation is dependent on UBC9.

Akt is localized in both cytosol and nucleus (13). We examined the subcellular localization of SUMOylated Akt using a yellow fluorescent protein (YFP)-based BiFC assay (30). Our data showed that most of the SUMOylated Akt is localized in the nucleus (Fig. 2D). Similar results were obtained from a subcellular fraction assay (Fig. 2E). These data indicate that SUMOylated Akt is mainly localized in the nucleus.

**Promotion of Akt SUMOylation by PIAS1 and deSUMOylation of Akt by SENP1**

SUMOylation is promoted by E3 ligases (4). PIASs family proteins, including the PIAS1, PIAS3, PIAS4 (also known as PIASy), PIASXα, and Χβ, are important E3s and responsible for the SUMOylation of various proteins (4, 31, 32). To determine whether SUMOylation of Akt is mediated by PIAS E3 SUMO ligase, we coexpressed 5 PIAS E3s with Akt in HEK293T cells and the SUMOylation was monitored. As shown in Fig. 2F, coexpression of PIAS1 showed strong increase in Akt SUMOylation. Moreover, both ectopic expressed and endogenous PIAS1 interact with Akt as measured by reciprocal immunoprecipitation and immunoblotting analyses (Fig. 2G and S2). Knockdown of the endogenous PIAS1 attenuated the SUMOylation of Akt (Fig. 2H). These data indicated that PIAS1 is a functional E3 ligase for Akt SUMOylation.

SUMOylation is a reversible process catalyzed by SENP family protease (4). Six Sumo-specific proteases (SENP1–SENP3 and SENP5–SENP7) have been reported (4). To identify the SENPs required for Akt deSUMOylation, we coexpressed SENPs with Akt and SUMO1, and SUMOylation of Akt in vivo was examined under denaturing conditions. As shown in Fig. 2I, expression of SENP1, but not other SENPs, markedly reduced the Akt SUMOylation. DeSUMOylation of Akt is dependent on the enzymatic activity of SENP1 (Fig. 2J). Our data showed that both ectopically expressed and endogenous SENP1 interacted with Akt (Fig. 2K and Supplementary Fig. S2C). Thus, we conclude that SENP1 is a protease for Akt deSUMOylation.

**K276 is the major SUMOylation site in Akt**

To study the biologic consequences of Akt SUMOylation, we first aimed to identify the SUMO acceptor site in Akt. SUMOylation typically occurs on lysine residues in a conserved consensus sequence ψKXE/D or E/DXXψ (4). Our data showed that multiple SUMOylated Akt bands could be detected when Akt1 was coexpressed with SUMO1 (Fig. 2A). As SUMO1 is not thought to form polymeric chain in vivo (4), we hypothesized that Akt1 could be SUMOylated at multiple sites. Among 34 lysine residues of Akt, at least 5 of them are potential SUMOylation sites, which distributes in PH, kinase, and C terminal domain, identified using SUMOplot analyses program (Fig. 3A and S3A). Our data showed that all 3 domains could be SUMOylated in cells (Fig. 3B). Then, we replaced individual predicted SUMOylation lysine to arginine and the SUMOylation was monitored. As shown in Fig. 3C, similar results were observed from various combinations of two lysine mutants (Supplementary Fig. S3B). Surprisingly, mutation of all 5 potential SUMOylation sites still did not completely abolish Akt SUMOylation (Fig. 3C). This suggested that Akt is localized in both cytosol and nucleus (13). We examined the subcellular localization of SUMOylated Akt using a yellow fluorescent protein (YFP)-based BiFC assay (30). Our data showed that most of the SUMOylated Akt is localized in the nucleus (Fig. 2D). Similar results were obtained from a subcellular fraction assay (Fig. 2E). These data indicate that SUMOylated Akt is mainly localized in the nucleus.
Figure 2. Akt1 is SUMOylated in vivo and in vitro. A, modification of Akt1 by SUMO1. Cells were transfected as indicated, and SUMOylation was conducted using an in vivo SUMOylation assay. SUMOylation of Akt was measured using an anti-Akt antibody. B, insulin stimulation increased the SUMOylation of endogenous Akt. H1975 cells were stimulated with or without insulin for 10 minutes, and SUMOylation of endogenous Akt was examined. C, depletion of UBC9 reduces SUMOylation of Akt1. H1299 cells were transfected with siRNA against UBC9. Then, Akt1 and His-SUMO1 were cotransfected and SUMOylation of Akt1 was analyzed. D, SUMOylated Akt1 is localized in the nucleus as detected using BiFC assay. E, SUMOylated Akt1 is localized in the nucleus as detected using a cell fractionation assay. F, PIAS1 enhances SUMO1 modification of Akt1. (Continued on the following page.)
mutants with only one lysine added on Akt-34KR and their SUMOylations were examined. Our data showed that Akt can be SUMOylated at K30, K64, K182, K189, K276, K111, but not K39 and K179 (Fig. 3F). As K168R and K183R reduced the kinase activity of Akt, we also measured whether Akt can be SUMOylated at K168R and K183R. Our data from one lysine Akt mutant showed that Akt could not be SUMOylated at K168 and K183 (Supplementary Fig. S3D). Collectively, these results indicated that Akt is SUMOylated at multiple lysine residues.

Although Akt is SUMOylated at multiple sites, we noticed that the integrity of residue K276 appeared to be particularly important because mutants carrying K276R, K276A, and K276Q substitution significantly reduced the levels of SUMOylated Akt (Fig. 3C and Supplementary Fig. S3E). To confirm that K276 is the major SUMOylation site of Akt, we mutated E278, which is localized in SUMOylation consensus and supposed to be essential for SUMOylation as well. As expected, mutation of E278 to alanine (A), glutamine (Q), or serine (S) markedly reduced Akt SUMOylation (Fig. 3G and Supplementary Fig. S3F). Thus, we conclude that K276 is one of the major SUMO acceptor site in Akt1.

**Mutation of K276 abolishes Akt kinase activity**

As the K276 is the main acceptor site for SUMO in Akt, we next aimed to examine the effect of SUMOylation on Akt kinase activity. First, we examined whether SUMOylation at K276 is required for Akt phosphorylation at T308 and S473. Consistently with previous results, K276R or E278A mutant had little effect on the phosphorylation of T308 and S473 (Fig. 4A). As it is known that phosphorylation of Akt at T308 and S473 requires Akt to be translocated to the membrane (13), these data suggest that K276 is not required for Akt membrane recruitment. Recent study showed that phosphorylation of T450 is essential for the folding and stability of Akt (33). Our data showed that K276R and E278A Akt showed similar phosphorylation at T450 with wild-type Akt (Fig. 4A), suggesting that folding of Akt is not affected by K276R and E278A mutation.

We also measured whether SUMOylation affects Akt activity using an in vitro kinase assay. Our data showed that neither K276R nor E278A was able to phosphorylate GSK3 in vitro (Fig. 4A). This was confirmed using an in vivo phosphorylation assay. As shown in Fig. 4B, expression of wild-type, but neither K276R nor E278A Akt, increased the phosphorylation levels of coexpressed GSK3 in cells cultured with serum. These data suggest that SUMOylation at K276 is essential for Akt activity.

To confirm that SUMOylation is required for Akt activity, we coexpressed SUMO1 and PIAS1 with Akt. The Akt activity was measured using an in vitro kinase assay. As shown in Fig. 4C, coexpression of PIAS1 and SUMO1 increased Akt activity. On the other hand, coexpression of SENP1, which inhibited the Akt SUMOylation, reduced Akt activity (Fig. 4D). Because K168R and K183R significantly reduced Akt kinase activity, we examined whether mutation of K168 or K183 affects Akt SUMOylation. Our data showed that both K168R and K183R reduced the SUMOylation of Akt (Supplementary Fig. S4), suggesting K168 and K183 are important to Akt kinase activity as well as SUMOylation.

To examine whether SUMOylation of Akt affects endogenous Akt activity, the expression of UBC9 or PIAS1 was knocked down and the phosphorylation of Akt substrates was examined using an anti-phosphorylated Akt substrate antibody. Our data showed that knockdown of UBC9 and PIAS1 reduced the phosphorylated levels of Akt substrates (Fig. 4E). The reduced phosphorylated FOXO protein levels in both H1975 and H1299 cancer cells corroborated this result (Fig. 4F and S5A). Moreover, knockdown of UBC9 or PIAS1 reduced the insulin-stimulated Akt activation (Fig. 4G) and levels of phosphorylated FOXO proteins (Supplementary Fig. S5B and S5C). Collectively, our data suggest that SUMOylation at K276 is essential for Akt activity.

We also examined whether mutation of K276 affects its ability to bind to substrates. We coexpressed Flag-tagged wild-type, K276R, and E278A Akt with HA-tagged GSK3, and their interaction was monitored using a co-immunoprecipitation assay. HA-GSK3 can be coprecipitated by all Akt forms (Supplementary Fig. S6A), indicating that SUMOylation at K276 is not required for Akt binding to its substrates. It has been shown that Akt can form dimer and the dimerization of Akt contributes to its activity as well (34). Our data showed that mutation of K276 did not affect the dimerization of Akt (Supplementary Fig. S6B). Moreover, mutation of K276 did not affect the TRAF6-mediated Akt ubiquitination (Supplementary Fig. S6C). The effect of SUMOylation on Akt nuclear localization was also examined. Both K276R and E278A showed similar subcellular localization with wild-type Akt (Supplementary Fig. S7A). Knockdown of UBC9 or PIAS1 had little effect on the subcellular localization of endogenous Akt (Supplementary Fig. S7B).

**SUMOylation of Akt is required for cell growth and tumorigenesis**

Akt plays essential roles in cell growth (13). Thus, we measured whether SUMOylation at K276 is involved in cell growth. Expression of wild-type Akt slightly increased cell growth of H1299 or MEF cells (Fig. 5A and B). In contrast, expression of K276R or E278A significantly reduced the cell growth compared with control when measured using an MTT assay (Fig. 5A and B). Similar results were obtained when cell...
proliferation was measured using an EdU incorporation assay (Fig. 5D). Moreover, expression of wild-type, but not K276R or E278A mutant, significantly promoted the cell proliferation of Akt-deficient MEFs (Fig. 5C). Given that Akt can promote cancer cell migration (13, 35), the effect of K276 SUMOylation on cell migration was also measured using a wound-healing assay. Our data showed that both K276R and E278A mutant inhibited cell migration (Fig. 5E). Together, we conclude that K276R and E278A can function as dominant-negative forms to block cell growth, proliferation, and cell migration.

It has been reported that Akt promotes cell survival by phosphorylating FOXO family of transcription factors (36).
Thus, we measured phosphorylation of FOXOs by wild-type, K276R, or E278A mutant Akt. Our data showed that expression of wild-type Akt increased the phosphorylation of FOXO. In contrast, expression of either K276R or E278A Akt suppressed FOXO phosphorylation (Fig. 5F).

It has been reported that E17 of Akt is frequently mutated to K (E17K) in various patients with cancer (37). E17K Akt exhibited an increased phosphorylation at T308 and it possesses stronger oncogenic activity. As expected, expression of E17K Akt in A549 cells exhibited an increased phosphorylation at T308 and it possesses stronger oncogenic activity (37). Interestingly, we found that SUMOylation of E17K is much stronger than wild-type Akt (Fig. 6A). Mutation of K276 or E278 markedly reduced Akt SUMOylation. Akt can be SUMOylated when only K276 is present, supporting that K276 is indeed a SUMO acceptor site of Akt. We found that mutation of K276 significantly reduced Akt SUMOylation. Akt can be SUMOylated when only K276 is present, supporting that K276 is indeed a SUMO acceptor site in vivo. Moreover, our study indicated that K276 is also essential to the activity of E17K. E17K mutation showed strong oncogenic activity. As expected, expression E17K Akt in A549 cells significantly promoted tumorigenesis (Fig. 6D–G). In sharp contrast, K276R or E278A mutant lost the ability to promote tumorigenesis (Fig. 6D–G). Collectively, these data suggested that SUMOylation of Akt at K276 is essential for Akt-mediated tumorigenesis.

Discussion

As an essential protein kinase involved in cell proliferation and tumorigenesis, Akt has been reported to be modified by phosphorylation, ubiquitination, and acetylation (13). In the current study, we found that Akt is also modified by SUMOylation at multiple sites. K276 is one of the major acceptor sites for SUMOylation. Importantly, SUMOylation is required for its kinase activity and mediated cell proliferation.

Our study indicated that K276 is the major SUMO acceptor site of Akt. We found that mutation of K276 significantly reduced Akt SUMOylation. Akt can be SUMOylated when only K276 is present, supporting that K276 is indeed a SUMO acceptor site in vivo. Recent study indicates that SUMO and ubiquitin can form a hybrid chain and modify target protein in a heterologous way (38). Thus, it is also possible that K276 is an acceptor site for the heterologous modification of SUMO/ubiquitin or other ubiquitin-like modifications. Moreover, we cannot exclude the potential modification of Akt by endogenous SUMO2/3.

Our study indicated that K276 is essential for Akt kinase activity. Mutation of K276 to Ala, Arg, or Gln completely abolished Akt kinase activity. As K276 is located in the central
kinase domain of Akt, several lines of evidence support that SUMOylation may play important roles in Akt activity: (i) mutation of E278, which is essential for Akt SUMOylation, impairs Akt kinase activity; (ii) coexpression of SUMO or Pias1, which may increase Akt SUMOylation, promoted Akt kinase activity; (iii) knockdown of Ubc9 or Pias1 reduced the kinase activity; and (iv) mutation of K276 did not affect its dimerization or binding to its substrate. Although we still do not know the exact mechanism that how SUMOylation affects Akt activation, our data strongly support that SUMOylation may be a novel mechanism to regulate Akt kinase activity. Interestingly, we also noticed that Akt may be SUMOylated at sites other than K276. Thus, SUMOylation may also affect other cellular functions of Akt.

Accumulating evidence indicated that SUMOylation can modify the translocation of eukaryotic proteins (4, 5). Akt is localized at plasma membrane, cytosolic, or nuclear (13). Akt is usually phosphorylated at plasma membrane and then the activated Akt is translocated to the nucleus (13). Interestingly, our data indicate that SUMOylated Akt is mainly localized in...
the nucleus. The underlying mechanism by which SUMOylated Akt is mainly localized in the nucleus is still unclear and needs further investigation.

Akt E17K mutation is a cancer-derived mutation, which has been found in various cancers (37, 39, 40). It has been shown that E17K mutation shows increased phosphorylation at T308 and S473, kinase activity, membrane localization, and ability to transform cells (37). A recent study indicates that ubiquitination of E17K by TRAF6, which is required for Akt activation, is stronger than that of wild-type Akt. In this study, we found that Akt E17K mutation shows stronger SUMOylation than wild-type Akt. Thus, our data suggest that the increased activation of E17K is through multiple mechanisms.

While our manuscript is under review, another study from Dr. Kirschner’s group showed that Akt1 is modified by SUMO1 (41). This is consistent with our findings that Akt is

Figure 6. SUMOylation of Akt is required for E17K Akt–mediated cell proliferation and tumorigenesis. A, SUMOylation of E17K mutant is increased compared with wild-type Akt. B, K276R or E278A mutant reduced the SUMOylation of E17K Akt. C, K276R or E278A mutant impaired the tumor growth in nude mice. A549 cells infected with the indicated Akt constructs were injected subcutaneously into nude mice. The tumors were harvested 1 month after injection. E, tumors were harvested 1 month after injection and the pictures were taken. F, weight of tumor in E was measured. Data in the graph represent the mean ± SD (n = 5; **, P < 0.01). G, size of the tumors was measured every 1 week. Five mice were inoculated in each group, and data in the graph represent the mean ± SD (n = 5; **, P < 0.01).
SUMOylated both in vivo and in vitro. However, the biologic functions or the underlying mechanism of this modification have not been investigated in their study. More interestingly, they found that more than 68% protein kinases they detected are modified by SUMO1 or SUMO2/3 (41). Together with these findings, our study suggests that SUMOylation may be a common and important modification for the activity and biologic functions of various protein kinases.

Recent evidence indicated that PTEN, a key regulator of Akt, is also SUMOylated (42, 43). SUMOylation is essential for PTEN biologic functions of various protein kinases.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: R. Li, P. Wang
Development of methodology: R. Li, J. Wei, C. Jiang, D. Liu, K. Zhang

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3. Yang XJ, Seto E. Lysine acetylation: codification of PTEN dephosphorylates PIP3 is also SUMOylated (42, 43). SUMOylation is essential for PTEN biologic functions of various protein kinases.

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): R. Li, J. Wei, C. Jiang, D. Liu, L. Deng
Analysis and interpretation of data (e.g., statistical analysis, bios-statistics, computational analysis): R. Li, J. Wei, C. Jiang, D. Liu, P. Wang
Writing, review, and/or revision of the manuscript: R. Li, J. Wei, C. Jiang, P. Wang
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): R. Li, D. Liu, L. Deng
Study supervision: R. Li, P. Wang

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