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ORIGINAL ARTICLE

SUMOylation regulates AKT1 activity

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Serine threonine kinase AKT has a central role in the cell, controlling survival, proliferation, metabolism and angiogenesis. Deregulation of its activity underlies a wide range of pathological situations, including cancer. Here we show that AKT is post-translationally modified by the small ubiquitin-like modifier (SUMO) protein. Interestingly, neither SUMO conjugation nor activation of SUMOylated AKT is regulated by the classical AKT targeting to the cell membrane or by the phosphoinositide 3-kinase pathway. We demonstrate that SUMO induces the activation of AKT, whereas, conversely, down-modulation of the SUMO machinery diminishes AKT activation and cell proliferation. Furthermore, an AKT SUMOylation mutant shows reduced activation, and decreased anti-apoptotic and pro-tumoral activities in comparison with the wild-type protein. These results identify SUMO as a novel key regulator of AKT phosphorylation and activity.

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INTRODUCTION

Increased activity of AKT also known as, Protein Kinase B (PKB) is one of the most common alterations observed in human cancer.[1] However, this hyperactivation of AKT is frequently not the result of mutation in the gene encoding this kinase. AKT has an amino-terminal pleckstrin homology (PH) domain, which is essential for binding phosphatidylinositol (3,4,5)-trisphosphate, a lipid second messenger produced by phosphoinositide 3-kinase (PI3K), the upstream activator of the signaling cascade. The PH domain is followed by an α-helical linker and a C-terminal kinase domain, critically controlled by phosphorylation.[2] In unstimulated cells, AKT localizes to the cytosol, where it is maintained in an inactive conformation through the interaction between its PH and kinase domains.[3] In the presence of proliferative signals, PI3K is activated and generates phosphatidylinositol (3,4,5)-trisphosphate that serves for the docking of AKT to the plasma membrane through binding to its PH domain. Membrane recruitment results in a conformational change that separates the PH and the kinase domains, unmasking two regulatory residues in AKT, threonine 308 and serine 473. Phosphorylation of these two residues is required for maximal activation of the kinase.[4]

The increased activity of AKT in cancer cells provides survival signals, support higher proliferative rates, alters metabolism and promotes cell migration. Tumor cells frequently depend on this high AKT activity for survival and therefore, understanding AKT activation and its regulation are important for the creation of better therapies to treat cancer.

SUMOylation is an important post-translational modification that has a pivotal role in a myriad of biological functions, including DNA damage response, cell growth, innate immune response, etc.[5] SUMOylation involves the covalent attachment of a member of the small ubiquitin-like modifier (SUMO) family of proteins to lysine residues in specific target proteins via an enzymatic cascade analogous to, but distinct from, the ubiquitylation pathway.[6] In this report, we show that AKT can conjugate to SUMO *in vitro* and *in vivo*. We have identified the lysine residues in AKT that work as main SUMO acceptors. We found that SUMOylation of AKT is not affected by its targeting to the cell membrane nor responds to the PI3K pathway, but it can be stimulated by cellular stress and it is negatively modulated by the promyelocytic leukemia (PML) tumor suppressor protein. Furthermore, we show that SUMO favors the activation of the protein, whereas Ubc9 down-modulation has the opposite effect. Finally, we demonstrate that an AKT SUMOylation mutant presents reduced activation that leads to a diminished anti-apoptotic activity, and a weaker oncogenic ability when compared with the wild-type (WT) protein. In summary, here we identify SUMO as a new crucial AKT kinase coactivator.

RESULTS

AKT1 is modified by SUMO

During the course of our previous studies on the SUMOylation of the phosphatase and tensin homolog (PTEN),[7] we noticed that AKT could also be found SUMOylated. Therefore, we proceeded to evaluate the putative conjugation of AKT to SUMO. *In vitro* SUMOylation assays were done using *in vitro* translated [^{35}S] methionine-labeled AKT1 protein, as a substrate. We detected AKT1 protein as a single band of the expected 60 kDa predicted molecular weight. When the reaction was incubated with SUMO1, SUMO2 or SUMO3, we observed higher-molecular-weight bands of around 75–80 and 90–95 kDa (Figure 1a, left panel), indicating that AKT1 is modified by SUMO1, SUMO2 and SUMO3 *in vitro*. To further prove that the bands correspond to AKT1-SUMO conjugates, we incubated AKT1-SUMO1 protein with the recombinant SUMO-specific protease SENP1. The high-molecular-weight bands detected in the lane corresponding to AKT1-SUMO1

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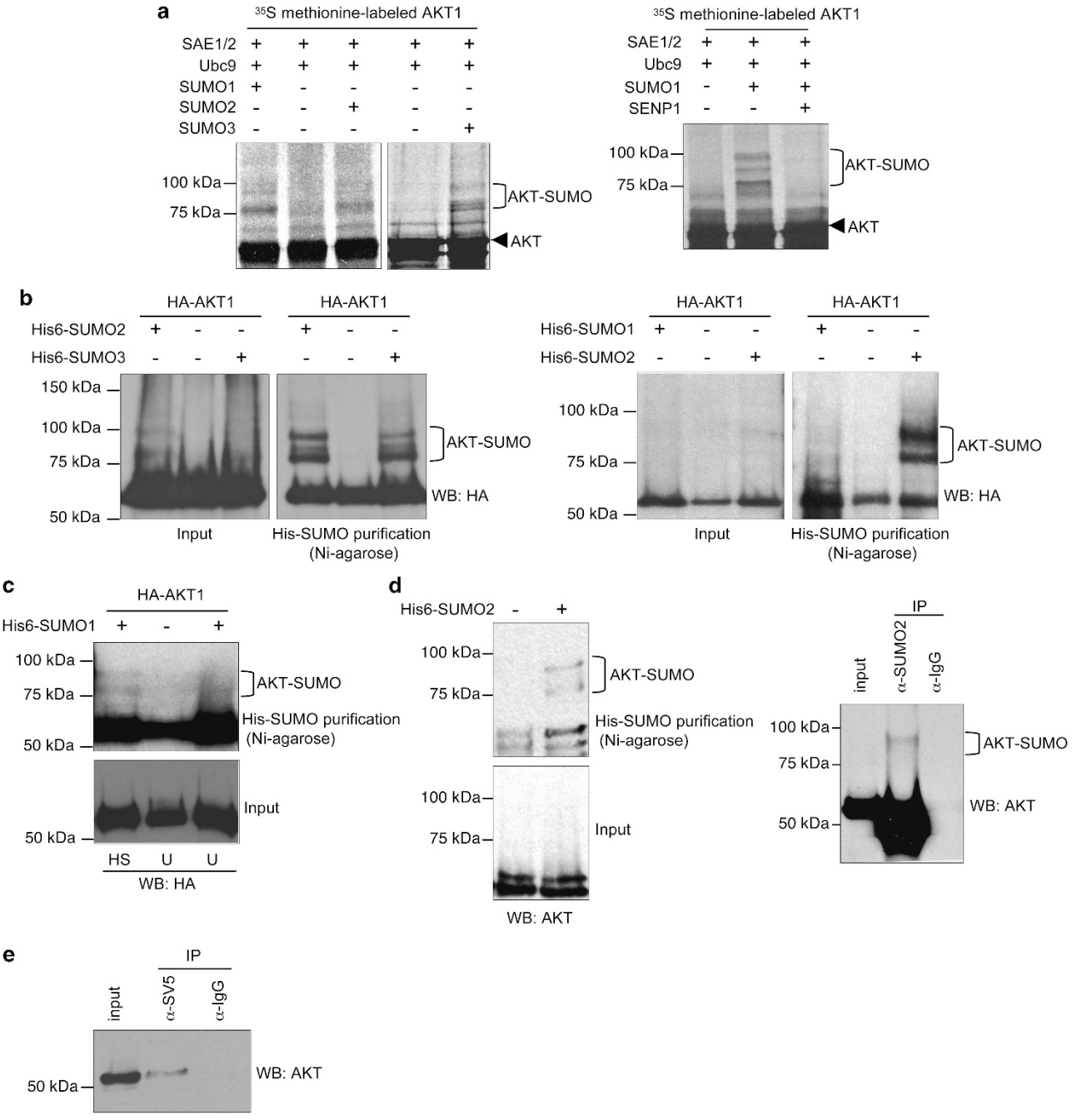

Figure 1. Covalent modification of AKT1 by SUMO1, SUMO2 and SUMO3 *in vitro* and *in vivo*. (**a**) *In vitro* translated [^{35}S]-labeled AKT1 was subjected to *in vitro* SUMOylation assay in the presence of SUMO1, SUMO2 or SUMO3 (left panel). SUMO1-conjugated AKT1 protein was then incubated with or without SENP1 as described in Materials and Methods (right panel). Proteins were resolved by SDS–polyacrylamide gel electrophoresis (PAGE) and visualized by autoradiography. (**b**) AKT1 is modified by SUMO2 and SUMO3 *in vivo*. HEK-293 cells were co-transfected with HA-AKT1 together with pcDNA, Ubc9 and His6-SUMO1, Ubc9 and His6-SUMO2 or Ubc9 and His6-SUMO3. Whole protein extracts or histidine-tagged purified proteins were analyzed by western blot (WB) analysis using anti-HA antibody. (**c**) Modification of transfected HA-AKT1 by SUMO1 in HEK-293 cells after heat stress (HS). HEK-293 cells were co-transfected with HA-AKT1 together with pcDNA or Ubc9 and His6-SUMO1 and 36 h after transfection cells were heat stressed by incubation at 45 °C for 20 min. Whole protein extracts or histidine-tagged purified proteins were analyzed by western blot analysis using anti-HA antibody. U, untreated cells. (**d**) Modification of endogenous AKT by SUMO2/3 in HEK-293 cells. HEK-293 cells were transfected with pcDNA or Ubc9 and His6-SUMO2. Whole protein extracts or histidine-tagged purified proteins were analyzed by western blot analysis using anti-AKT antibody (left panel). Protein extracts from untransfected HEK-293 cells were subjected to immunoprecipitation (IP) with anti-SUMO2/3 antibodies or with IgG control, as indicated. Immunoprecipitated samples were run on a SDS–PAGE gel and were analyzed by western blot analysis using anti-AKT antibody (right panel). (**e**) Co-immunoprecipitation of endogenous AKT with transfected SV5-Ubc9 in HEK-293 cells. Protein extracts from HEK-293 cells transfected with SV5-Ubc9 were subjected to immunoprecipitation with anti-SV5 antibody or with IgG control, as indicated. Immunoprecipitated samples were run on a SDS–PAGE gel and were analyzed by western blot using anti-AKT antibody.

disappeared after incubation of the reaction with SENP1 (Figure 1a, right panel). All together, these results demonstrate that AKT1 is SUMOylated *in vitro*. In addition, the presence of several bands corresponding to AKT1-SUMO1 in the *in vitro* assay indicates that SUMOylation occurs at more than one site. Then, in order to determine whether AKT1 also conjugates to SUMO within the cell, HEK-293 cells were co-transfected with HA-tagged AKT1 together with Ubc9 and His6-tagged SUMO1, SUMO2, SUMO3 or pcDNA plasmids. At 48 h after transfection, His6-tagged proteins were purified in denaturing conditions using nickel beads. Western blot analysis of the purified extracts with anti-HA antibody revealed bands of the expected size corresponding to AKT1-SUMO proteins only in those cells co-transfected with His6-SUMO2 or His6-SUMO3 (Figure 1b, left panel), indicating that AKT1 is modified by SUMO2 and SUMO3 *in vivo*. However, we could not detect the expected AKT1-SUMO1 bands in those cells transfected with His6-SUMO1 (Figure 1b, right panel), despite repeated attempts and using different cell lines, suggesting AKT1 is not modified by SUMO1 *in vivo* under these conditions. It has been previously described that some stress conditions can induce SUMO conjugation,[8–12] therefore we decided to evaluate the conjugation of AKT1 to SUMO1 in stressed cells. HEK-293 cells were co-transfected with HA-AKT1 and pcDNA or His6-SUMO1 and Ubc9, and 36 h after transfection, cells were heat stressed by incubation at 45 °C for 20 min. Whole-cell extracts and histidine-tagged purified proteins were then analyzed by western blot analysis. As shown in Figure 1c, we could not detect AKT1-SUMO1 in the untreated cells, as previously observed. However, we can see clear AKT1-SUMO1 bands in the stressed cells. These results demonstrate that AKT1 could be conjugated to SUMO1 *in vivo* under stress conditions. Furthermore, similar experiments done with SUMO2 revealed an increase in the AKT1 conjugation to SUMO2 under heat-shock stress (Supplementary Figure 1). To confirm that endogenous AKT protein is modified by SUMO2, total protein extracts and His-tagged purified proteins obtained from HEK-293 cells transfected with His6-SUMO2 and Ubc9 were analyzed by western blot analysis using anti-AKT antibody. Bands of the expected size corresponding to AKT-SUMO2 protein were detected only in the cells transfected with His6-SUMO2 (Figure 1d, left panel), indicating that endogenous AKT protein can be modified by SUMO2. Modification of endogenous AKT protein by SUMO2/3 was also determined by immunoprecipitation analysis. Endogenous SUMO2/3 protein was immunoprecipitated from untransfected HEK-293 cells using anti-SUMO2/3 antibody and a band with a molecular weight similar to the upper AKT-SUMO2 band was detected using anti-AKT antibody (Figure 1d, right panel). SUMOylated proteins interact with the E2 SUMO-conjugating enzyme Ubc9 as part of the SUMOylation process. Thus, we decided to evaluate the putative interaction between Ubc9 and AKT. HEK-293 cells were transfected with SV5-Ubc9 and at 48 h we analyzed the co-immunoprecipitation of both proteins. As shown in Figure 1e, endogenous AKT co-immunoprecipitated with Ubc9. All together, these data demonstrate that AKT can be conjugated to SUMO1, SUMO2 and SUMO3 *in vitro* and *in vivo*.

Identification of the SUMO-modification sites in AKT1

The SUMOplot prediction system identified four putative SUMO consensus sites (K64, K182, K189 and K276) in AKT1. We constructed single or double AKT1 mutants in the putative lysine residues and analyzed their capability to conjugate to SUMO *in vitro*. We observed a clear reduction in SUMO2 conjugation in single mutant proteins in which we mutated individually lysines 64, 182 or 189 (AKT1-K64R, AKT1-K182R and AKT1-K189R, respectively) and in the double mutant in the lysine residues 182 and 189 AKT1-K182RK189R (Figure 2a). Similarly, we also observed a clear reduction in SUMO1 conjugation to AKT1-K64R and AKT1-K182RK189R (Supplementary Figure 2). These results pointed to the lysine residues 64, 182 and 189 as SUMO-acceptor sites in AKT1. To verify this information, we constructed a triple AKT1 mutant in the lysine residues 64, 182 and 189 (AKT1-SMUT) and then, we analyzed its capability to conjugate to SUMO *in vitro* and *in vivo*. We observed a reduction in SUMO1 conjugation to AKT1-SMUT in comparison with the WT protein (Supplementary Figure 2) and we did not detect any band corresponding to AKT1-SMUT protein conjugated to SUMO2 (Figure 2b) *in vitro*. Importantly, SUMO2 modification of AKT1-SMUT was decreased to almost undetectable levels *in vivo* (Figure 2c), indicating that lysine residues 64, 182 and 189 in AKT1 are implicated in the conjugation to SUMO.

SUMO increases the activity of AKT *in vitro* and *in vivo*

In order to evaluate the effect of SUMO conjugation on the AKT activity, we carried out an *in vitro* kinase assay using unmodified or SUMOylated recombinant AKT1 protein, and recombinant p27 protein as AKT substrate. As expected, unmodified AKT1 induced the phosphorylation of p27 (Figure 3a), whereas a clear increase in the levels of phosphorylated p27 was detected when we used AKT1 that had been previously subjected to an *in vitro* SUMOylation reaction in the presence of SUMO2 (Figure 3a) or SUMO1 (Supplementary Figure 3). These results indicated that SUMO increased the kinase activity of AKT1 *in vitro*. To determine whether SUMOylation regulates the activity of AKT *in vivo*, we evaluated the phosphorylation ratio of AKT-WT versus AKT-SMUT in cells. MCF-7 cells were transfected with pcDNA, HA-AKT1-WT or the SUMOylation mutant HA-AKT1-SMUT, and 24 h after transfection, cells were serum deprived for 24 h and stimulated or not with insulin. Transfected AKT1 protein was immunoprecipitated using anti-HA antibody and then western blot analysis using anti-phospho-AKT antibody was carried out. The ratio of phosphorylated/unphosphorylated SUMOylation mutant AKT1 protein (p-AKT1-SMUT/AKT1-SMUT) was clearly lower than that of the WT AKT1 protein (Figure 3b). In addition, although insulin treatment induced an increase in the p-AKT1-SMUT/AKT1-SMUT ratio, this fraction was clearly lower than the one observed in cells expressing the WT protein (Figure 3b). These results suggested that the SUMOylation of the AKT1 protein favored the activation of the protein. In order to confirm this hypothesis, we co-transfected MCF-7 cells with HA-AKT1-WT together with pcDNA or Ubc9 and SUMO2. At 24 h after transfection, cells were serum deprived for 24 h and then treated with insulin or wortmannin. Then, activation of AKT as well as of some AKT downstream targets was analyzed by western blot analysis. As shown in Figure 3c, the p-AKT/AKT ratio in the untreated or insuline-treated cells transfected with Ubc9 and SUMO2 was higher than the one detected in cells transfected with pcDNA. In agreement with these data, the phosphorylation of AKT substrates FOXO and p70 was increased by cotransfection of Ubc9 and SUMO2 (Figure 3c). These results indicated that SUMO induced the activation of AKT, both at uninduced and insuline-induced conditions.

We then decided to analyze the subcellular distribution of AKT after SUMO transfection. HeLa cells were transfected with HA-AKT1-WT in the presence or absence of Ubc9 and SUMO2 or with HA-AKT1-SMUT, and 48 h after transfection, we performed a cell fractionation assay. Western blot analysis using anti-HA antibody revealed that transfected AKT1 was mainly detected in the cytoplasm. In addition, we did not observe significant differences in the localization of WT and SUMOylation mutant AKT1 protein (Figure 3d and Supplementary Figure 3). However, transfection of SUMO2 induced a decrease in HA-AKT1 protein levels in the nucleus and the appearance of a band of the expected AKT-SUMO2 molecular weight in the cytoplasm (Figure 3d), indicating that the AKT-SUMO2 protein is mainly localized in the cell cytoplasm.

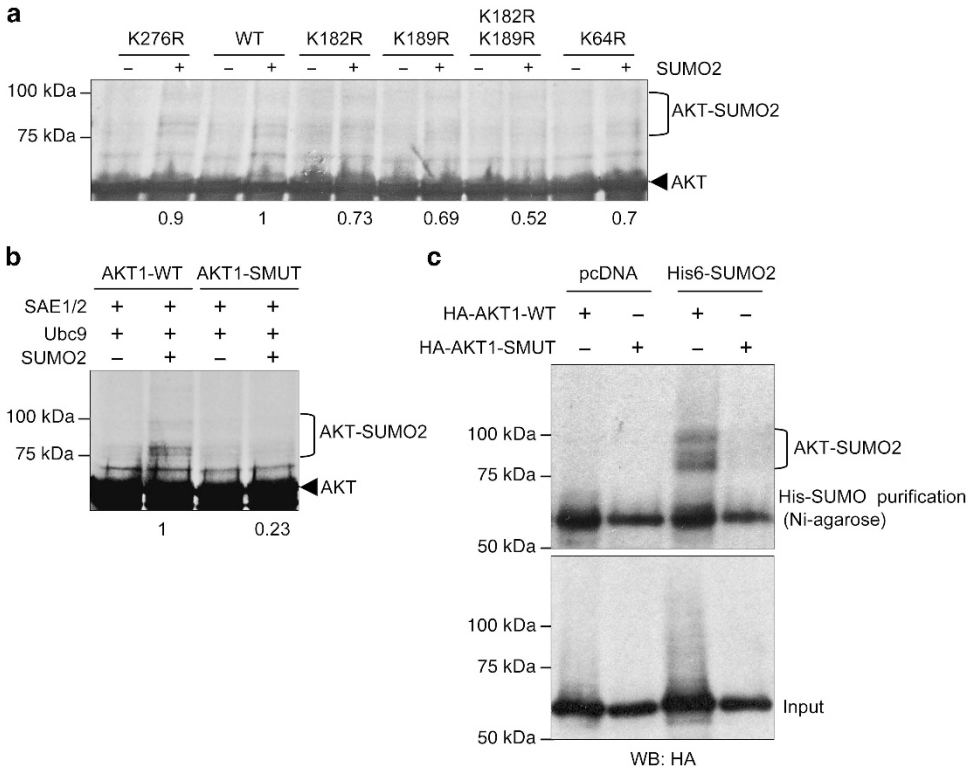

Figure 2. Identification of the lysine residues in AKT1 that conjugate SUMO. (**a**) *In vitro* translated [^{35}S]-labeled AKT1-WT or the indicated AKT1 mutants were subjected to *in vitro* SUMOylation assay in the presence of SUMO2. Proteins were resolved by SDS–polyacrylamide gel electrophoresis (PAGE) and visualized by autoradiography. Quantification of AKT1-SUMO/AKT1 ratios is shown. (**b**) *In vitro* translated [^{35}S]-labeled AKT1-WT or an AKT1 mutated in three lysine residues, as indicated, were subjected to *in vitro* SUMOylation assay in the presence of SUMO2. Proteins were resolved by SDS–PAGE and visualized by autoradiography. Quantification of AKT1-SUMO/AKT1 ratios is shown. (**c**) Modification of transfected HA-AKT1-WT or HA-AKT1-SMUT by SUMO2 in HEK-293 cells. HEK-293 cells were co-transfected with HA-AKT1-WT or HA-AKT1-SMUT together with pcDNA or Ubc9 and His6-SUMO2. Whole protein extracts or histidine-tagged purified proteins were analyzed by western blot (WB) analysis using anti-HA antibody.

Modulation of AKT SUMOylation and its activation

To clarify the mechanism by which SUMO activates AKT, we examined the putative phosphorylation of the AKT-SUMO protein and its regulation. Western blot analysis of histidine-tagged purified extracts obtained from HEK-293 cells co-transfected with HA-AKT1-WT together with pcDNA or His6-SUMO2 and Ubc9, revealed that a fraction of the conjugated AKT1-SUMO2 protein was phosphorylated (Figure 4a), indicating that AKT1-SUMO protein can be activated. Similar experiments were carried out using the constitutively active myristoylated AKT1 construct (Myr-AKT1-WT). As shown in Figure 4b, SUMOylation of AKT1 was not affected by the AKT targeting to the cell membrane. In addition, although the phosphorylation status of unSUMOylated AKT clearly increased after the targeting to the cell membrane, the levels of phosphorylated AKT-SUMO2 protein was not affected by myristoylation (Figure 4b). Then, we analyzed the levels of phosphorylated AKT-SUMO2 protein after modulation of the PI3K pathway. MCF-7 cells were co-transfected with HA-AKT1-WT and pcDNA or Ubc9 and His6-SUMO2, and 24 h after transfection, cells were serum starved for 24 h and treated with insulin or wortmannin. Then, histidine-tagged purified proteins were analyzed by western blot analysis. As shown in Figure 4c, SUMOylation of AKT occurred independently of the PI3K pathway and insuline treatment did not result in the induction of AKT-SUMO2 protein phosphorylation. Furthermore, we observed the presence of phosphorylated AKT-SUMO2 in those cells treated with wortmannin even when the levels of phosphorylated unSUMOylated AKT protein were undetectable (Figure 4c), suggesting that activation of AKT-SUMO protein occurs independently of the PI3K pathway. Finally, we could not observe a regulation of AKT SUMOylation after co-expression of the phosphatase PTEN (Supplementary Figure 4). All together, these results indicated that the SUMOylation of AKT is not dependent on the PI3K pathway and it is not regulated by its targeting to the cell membrane. In addition, these results also indicate that the activation of the SUMOylated-AKT protein is independent of PI3K activation.

We then wanted to analyze the putative interplay between AKT SUMOylation and ubiquitination. Proteasome inhibition induces the accumulation of mixed polyubiquitinated and polySUMOylated chains of some substrates.[13] Therefore, we decided to evaluate whether AKT-SUMO2 chains accumulated in cells treated with MG132. As shown in Figure 4d, AKT1-SUMO2 conjugation was not affected by proteasome inhibition, suggesting that SUMO2 conjugation does not work as a signal for polyubiquitination of AKT1.

Recently, it has been demonstrated that PML loss antagonizes p-AKT inactivation, leading to AKT activation and function.[14] The results shown here indicate that SUMO also favors the activation of AKT. We then decided to evaluate the effect of PML in the SUMOylation of AKT. HEK-293 cells were co-transfected with HA-AKT1-WT together with pcDNA or His6-SUMO2 and Ubc9, in the presence or absence of pcDNA-PML, and western blot analysis of the histidine-tagged purified proteins was carried out. The level of AKT1-SUMO2 protein was clearly reduced in cells transfected with PML (Figure 4e), indicating that PML negatively regulates the SUMOylation of AKT.

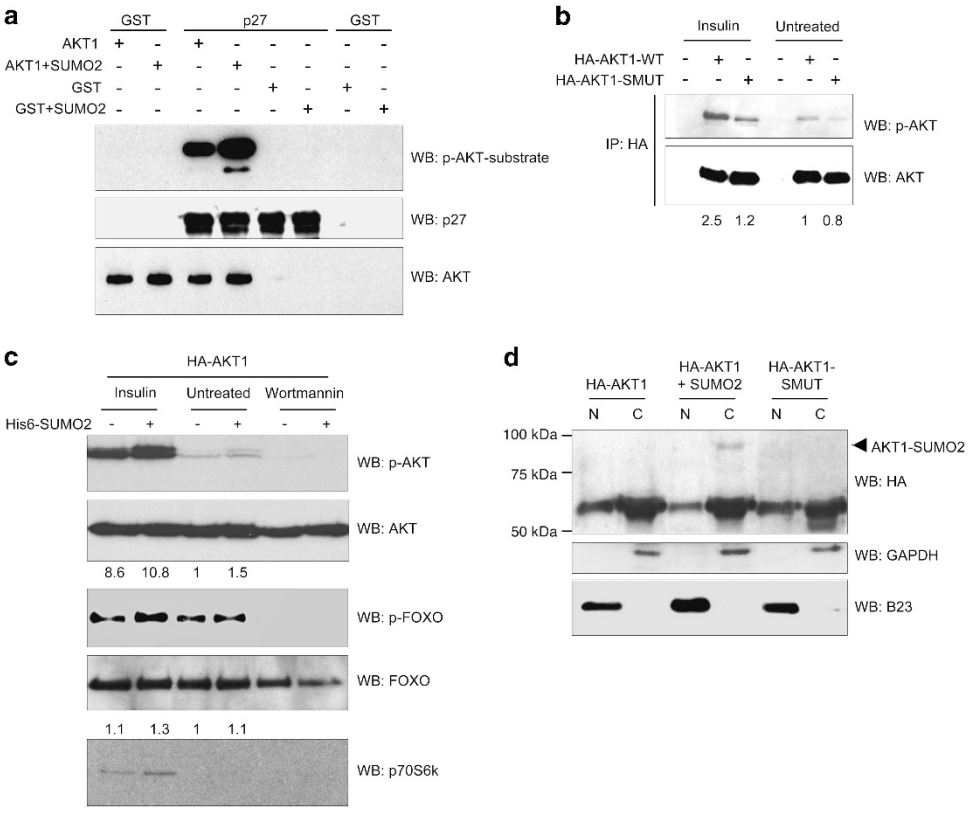

Figure 3. Activation of AKT by SUMO. (**a**) *In vitro* kinase assay using recombinant AKT1 protein subjected to *in vitro* SUMOylation assay in the presence or absence of SUMO2 and recombinant p27 protein as a substrate. Phosphorylation of p27 was detected using anti-phospho-AKT substrate antibody. Equal loading of p27 and AKT per reaction was confirmed by western blot (WB) analysis with the antibody directed against p27 or AKT protein. (**b**) Protein extracts from MCF-7 cells transfected with HA-AKT-WT or HA-AKT-SMUT and subjected to starvation-stimulation, as indicated, were subjected to immunoprecipitation with anti-HA antibody. Immunoprecipitated samples were run on a SDS–polyacrylamide gel electrophoresis and analyzed by western blot analysis with the indicated antibodies. Numbers indicate densitometrically determined p-AKT/AKT ratio. (**c**) Western blotting of lysates from MCF-7 cells co-transfected with HA-AKT1-WT and pcDNA or Ubc9 and His6-SUMO2 and subjected to starvation-stimulation-inhibition. Numbers indicate densitometrically determined p-AKT/AKT or pFOXO3a/FOXO3a ratios. (**d**) Fractionation of Hela cells transfected with the indicated plasmids. GAPDH and B23 were used as markers of the cytoplasmic (C) and nuclear (N) fractions, respectively.

SUMO contributes to the anti-apoptotic and pro-tumoral activity of AKT

Activated AKT protein favors cell proliferation and inhibits the apoptosis induced by different stimuli. As a first approach to test the putative effect of SUMO on cell proliferation and AKT activation, we decided to evaluate the consequences of Ubc9 down-modulation on the proliferative rate and on the phosphorylation status of AKT. Transfection of PC3 cells with siUbc9 significantly reduced the proliferation rate of PC3 cells (Figure 5a, left panel) and decreased the p-AKT/AKT ratio (Figure 5a, right panel). Furthermore, incubation of the transfected cells with wortmannin for 24 h abolished the proliferative advantage of the cells transfected with small interfering RNA control (Figure 5a, left panel). These results suggested a positive correlation between SUMOylation and AKT activation. Then, in order to clearly determine the contribution of SUMO to the proliferative and anti-apoptotic activity of AKT, we generated a SUMOylation mutant of the constitutively active AKT1 protein and evaluated its SUMOylation *in vivo*. As shown in Figure 5b, Myr-AKT1-WT protein was modified by SUMO2. However, we did not detect SUMOylation of the Myr-AKT1-SMUT protein (Figure 5b). In addition, we also observed that the p-AKT/AKT ratio detected in the cells transfected with Myr-AKT1-WT was clearly higher than the one detected in cells transfected with the mutant, Myr-AKT1-SMUT, suggesting again that SUMO contributes to the complete activation of AKT. Then, in order to evaluate the effect of SUMOylation on the anti-apoptotic activity of AKT1, we generated COS-7 cells that over-express Myr-AKT1-WT or Myr-AKT1-SMUT, and 24 h after serum deprivation, we evaluated the phosphorylation of AKT and exposed the cells to ultraviolet irradiation as a trigger of apoptosis.[15] The levels of p-AKT observed in the COS-7 cells expressing Myr-AKT1-SMUT were lower than the ones observed in the Myr-AKT1-WT-expressing cells (Figure 5c, left panel). In addition, and as expected, Myr-AKT1-WT inhibited ultraviolet-induced apoptosis in COS-7 cells (Figure 5c, right panel). In contrast, expression of Myr-AKT1-SMUT was ineffective in controlling ultraviolet-induced apoptosis (Figure 5c, right panel), indicating that SUMOylation contributes to the anti-apoptotic activity of AKT1.

Finally, in order to study the role of AKT1 SUMOylation in oncogenesis, we generated NIH-3T3 cells that over-express Myr-AKT1-WT or Myr-AKT1-SMUT, and 24 h after serum deprivation, we performed a focus formation assay. Consistent with previously published data showing that Myr-AKT1 can transform NIH-3T3 cells,[16–18] the expression of Myr-AKT1-WT induced the formation of foci in these cells, whereas the vector control cells failed to do so (Figure 5d, left panel). The number of foci induced by Myr-AKT1-SMUT was significantly lower than the one obtained in the cells expressing the WT protein, in agreement with the lower pAKT levels observed in the Myr-AKT1-SMUT-expressing cells (Figure 5d, right panel). These results indicated that SUMOylation contributes to the transforming activity of AKT1. All together, these data indicated that SUMOylation contributes to the proliferative and anti-apoptotic activity of AKT.

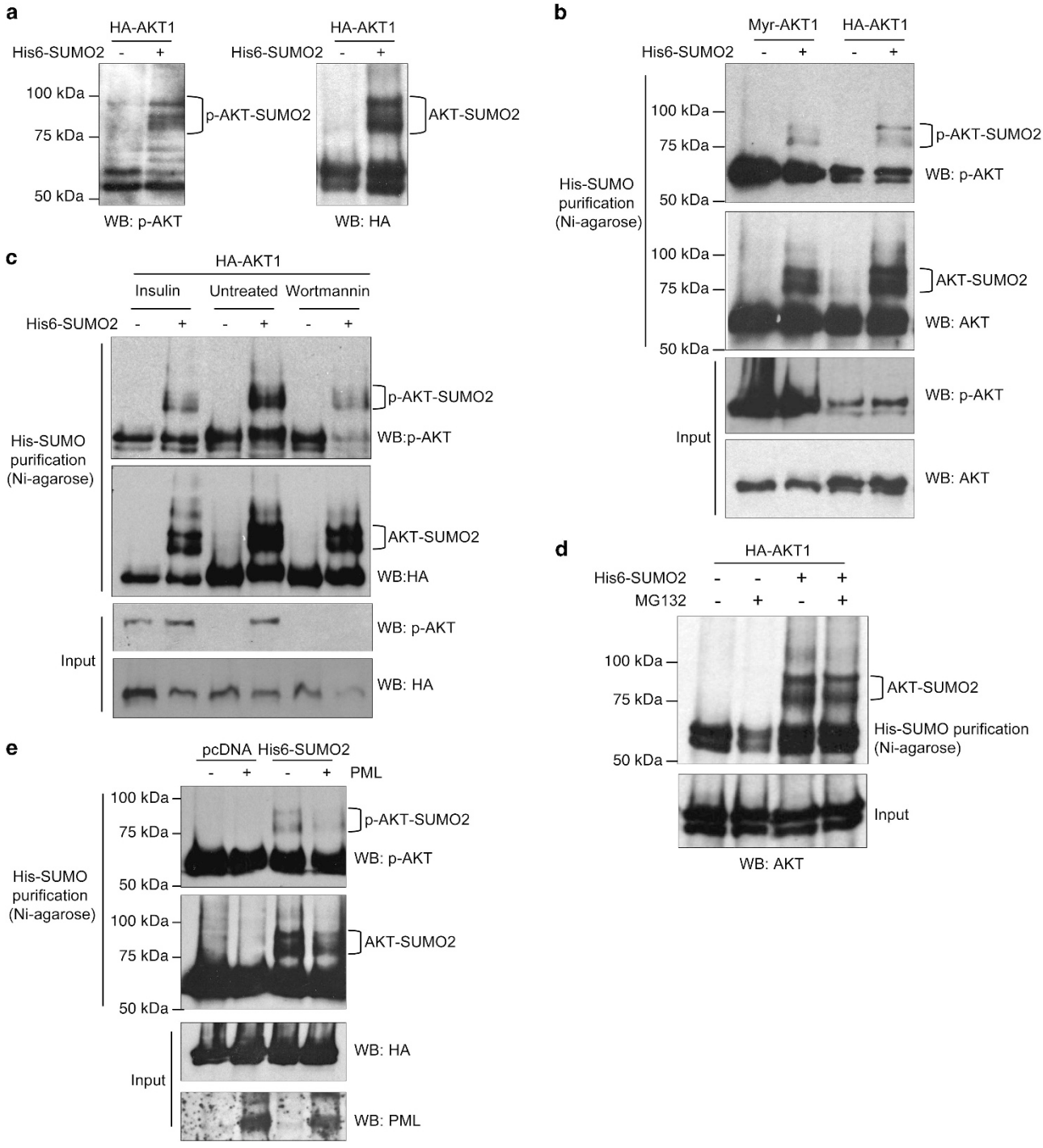

Figure 4. Modulation of AKT SUMOylation. (**a**) p-AKT in western blot (WB) analysis of AKT1-SUMO2. HEK-293 cells were co-transfected with HA-AKT1 together with pcDNA or Ubc9 and His6-SUMO2. Histidine-tagged purified proteins were analyzed by western blot analysis using anti-phospho-AKT (Ser-473) antibody or anti-HA antibody. (**b**) p-AKT in western blot analysis of Myr-AKT1-SUMO2 or AKT1-SUMO2. HEK-293 cells were co-transfected with Myr-AKT1 or HA-AKT1 and pcDNA or Ubc9 and His6-SUMO2. Whole protein extracts or histidine-tagged purified proteins were analyzed by western blot analysis using anti-phospho-AKT antibody and the same membrane was reblotted with anti-AKT antibody. (**c**) Western blot analysis of p-AKT-SUMO2 and AKT-SUMO2 in MCF-7 cells subjected to starvation-stimulation-inhibition. HEK-293 cells were co-transfected with HA-AKT1 together with pcDNA or Ubc9 and His6-SUMO2. After 24 h, cells were serum deprived for 16 h and then mock treated, stimulated with insulin or treated with wortmannin. Whole protein extracts or histidine-tagged purified proteins were analyzed by western blot analysis using anti-phospho-AKT or anti-HA antibodies. (**d**) Modification of AKT by SUMO2 after inhibition of the proteasome. HEK-293 cells were co-transfected with HA-AKT1 and pcDNA or Ubc9 and His6-SUMO2. After 36 h, cells were treated or not with MG132, as indicated. Whole protein extracts or histidine-tagged purified proteins were analyzed by western blot analysis using anti-AKT antibody. (**e**) Modification of AKT by SUMO2 after overexpression of PML. HEK-293 cells were co-transfected with HA-AKT1 together with pcDNA or Ubc9 and His6-SUMO2, and in the presence or absence of pcDNA-PML. Whole protein extracts or histidine-tagged purified proteins were analyzed by western blot analysis using the indicated antibodies.

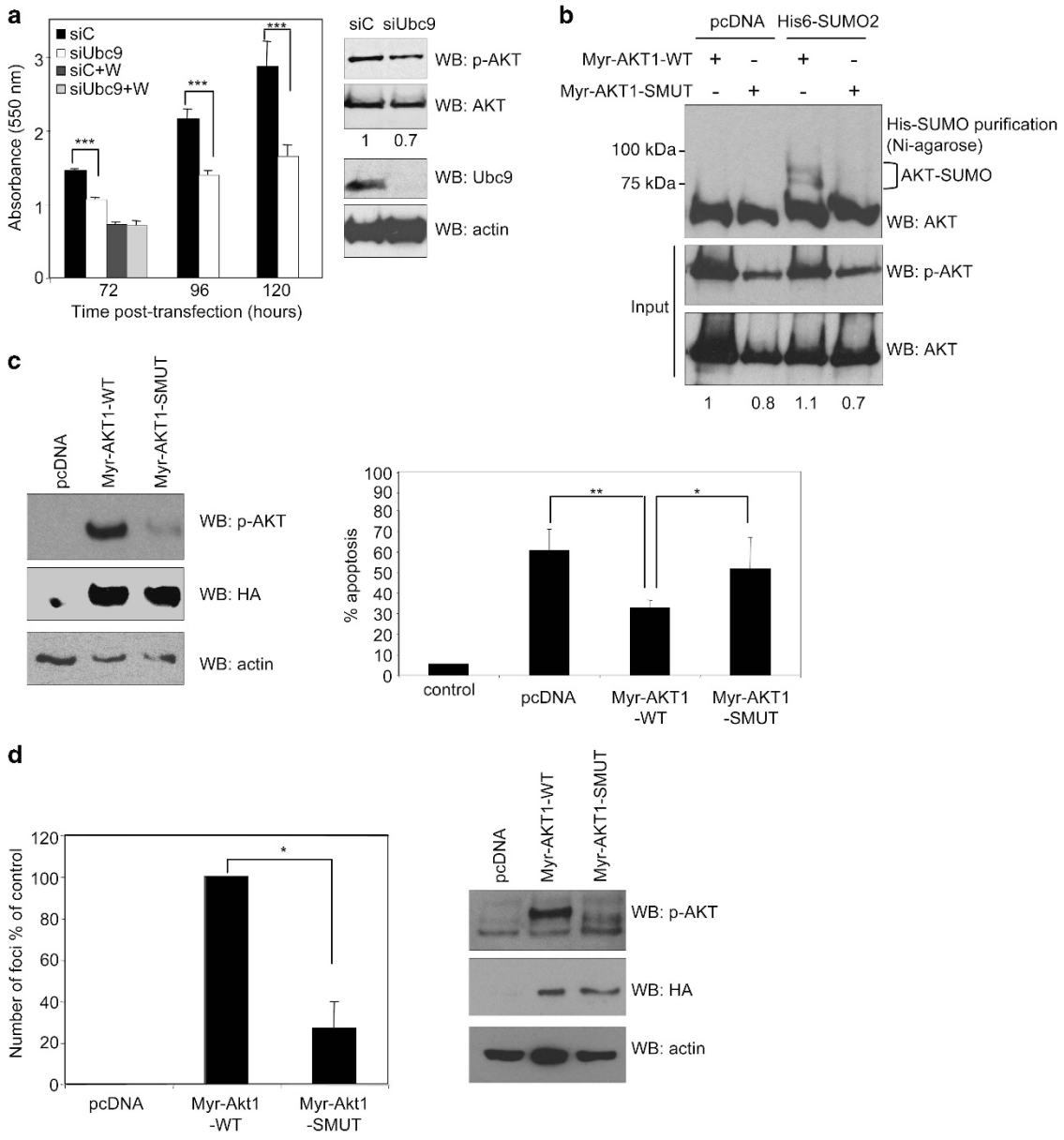

Figure 5. SUMO favors AKT activity. (**a**) Growth rates of PC3 cells transfected with siUbc9 or siC were monitored by the MTT assay (left panel) and starved-stimulated cells were analyzed by western blot (WB) analysis (right panel). In parallel, 48 h after transfection with siUbc9 or siC, cells were treated with wortmannin (W) for 24 h when MTT incorporation was evaluated. Error bars are s.d. of quintuplicates. Numbers indicate densitometrically determined p-AKT/AKT ratios. (**b**) Modification of transfected Myr-AKT1-WT or Myr-AKT1-SMUT by SUMO2 in HEK-293 cells. HEK-293 cells were co-transfected with Myr-AKT1 or Myr-AKT1-SMUT and pcDNA or Ubc9 and His6-SUMO2. Whole protein extracts or histidine-tagged purified proteins were analyzed by western blot analysis using the indicated antibodies. (**c**) Serum-starved COS-7 cells expressing Myr-AKT1 or Myr-AKT1-SMUT were analyzed by western blot analysis (left panel) and the percentage of apoptotic cells in response to ultraviolet irradiation was then determined by flow cytometry (right panel). The results are presented as mean of three independent experiments performed in triplicate, and error bars represent s.d. (**d**) Focus formation assay with NIH-3T3 cells expressing Myr-AKT1 or Myr-AKT1-SMUT (left panel). Serum-starved cells were analyzed by western blot analysis (right panel). Error bars are standard deviation (s.d.) of triplicates. **P*<0.05, ***P*< 0.005, ***P*<0.0005, Student´s test.

DISCUSSION

The results reported here reveal that AKT interacts with Ubc9, suggesting that AKT is a SUMO substrate. In agreement with this possibility, we demonstrate that AKT1 can be SUMOylated by SUMO1, SUMO2 and SUMO3 both *in vitro* and *in vivo*. However, although SUMO2 or SUMO3 conjugation of AKT1 *in vivo* was already detected in unstressed cells, AKT1-SUMO1 conjugation *in vivo* was detected only after heat-shock conditions. These results may indicate that AKT1 is not constitutively modified by SUMO1 and instead is only modified by SUMO1 after cells are exposed to stress conditions. However, a recent report on Arabidopsis revealed that heat-shock stress mostly increases the abundance of existing conjugates as opposed to modifying new targets.[12] Therefore, another possible explanation of the inability to detect AKT1-SUMO1 in unstressed cells is that the small fraction of AKT1 that is conjugated to SUMO1 at a specific time point is too low to be detected. In agreement with this hypothesis, we also observed an increase in the amount of AKT1-SUMO2 in cells exposed to heat-shock stress. Interestingly, exposure of

cells to heat shock has been reported to result in activation of AKT,[19,20] suggesting that SUMO may be implicated in this activation.

By using *in vitro* and *in vivo* SUMOylation assays on AKT1 mutants, we demonstrate that lysine residue 64 at the PH domain, which mediates lipid–protein and/or protein–protein interactions, and the lysine residues 182 and 189 at the kinase domain work as SUMO acceptors in AKT1. These three lysine residues are evolutionary conserved between mouse and human, with 64 and 189 lysine residues being present in the three isozymes of human AKT, and 182 in AKT1 and AKT3.

In vitro kinase assays using unmodified recombinant AKT1 or *in vitro* SUMOylated recombinant AKT1 protein demonstrated that SUMO increased the kinase activity of AKT1 *in vitro*. Similarly, SUMO potentiated the activation and activity of AKT in transfected cells. There are different mechanisms that may explain the activation of AKT by SUMO. One possibility would be that the interaction of SUMO with AKT strengthens its membrane association. Alternatively, SUMO could hinder the deactivation of AKT, as it has been proposed for the heat-shock protein 90.[21] However, the higher kinase activity of the AKT1-SUMO protein *in vitro* indicate the requirement of alternative explanations. The constitutive disengage of the PH domain from the kinase domain in AKT,[22] and the dimerization of the protein[23] have been proposed as mechanisms that induce AKT phosphorylation. It is well-known that SUMO attachment can induce a conformational change in the substrate,[24] that often has a positive effect on protein–protein interactions, and that can promote the assembly of several multi-protein complexes.[25] It is therefore possible to hypothesize that: the binding of SUMO to AKT may open the conformation of the kinase, leading to the activation of the protein; that SUMO may serve as a scaffold to recruit the adaptors for AKT, in turn facilitating AKT activation; or that SUMO may mediate the formation of homo- or hetero-multimeric protein complexes, leading to the activation of AKT.

SUMOylation of many substrates is regulated through a complex interplay with other post-translational modifications. For example, phosphorylation can modulate SUMOylation[26] and SUMOylation with SUMO2 can promote ubiquitilation.[27,28] We could not detect a regulation of the AKT SUMOylation by phosphorylation, and SUMO2 conjugation did not seem to act as a signal for the polyubiquitilation of AKT1. However, we identified PML as a negative modulator of AKT SUMOylation. It is then tempting to speculate that the previously described increased AKT activation associated with PML loss[14] could be related with the negative regulation of AKT SUMOylation by PML.

Supporting a role for SUMO as an AKT activator, we detected a decrease in cell proliferation and a reduction in the levels of activated AKT upon down-modulation of the E2 SUMO-conjugating enzyme Ubc9 in PC3 cells. Furthermore, a SUMOylation mutant of AKT showed reduced activation and compromised proliferative and anti-apoptotic activities.

In summary, here we provide evidences that SUMO interacts with and activates AKT. A deregulation of SUMO homeostasis has been shown to be associated with different types of cancer, such as prostate and breast cancer.[29] It is therefore plausible that SUMO may provide intrinsic survival signals in the absence of external stimulation for AKT activation or that a deregulation of the SUMO-AKT signaling pathway may underlie in human cancers. Importantly, this post-translational modification of AKT may also have consequences on therapeutic strategies as SUMO may limit the efficacy of PI3K/AKT pathway inhibitors. However, AKT-SUMO protein interaction may be an attractive target for the design of new anticancer drugs. In this sense, the specific targeting of the AKT-SUMO interaction may improve the efficacy of PI3K/AKT inhibitors and reduce the side effects resulting from inhibiting this pathway.

MATERIALS AND METHODS

Cell lines and plasmids

MCF-7, HEK-293, COS-7, HeLa, PC3 and NIH-3T3 cells were cultured in complete medium (Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1% penicillin). The cells were transiently transfected using Xtreme (Roche, Farma SA, Madrid, Spain) or lipofectamine (Invitrogen, Life Technologies SA, Madrid, Spain) transfection reagents, as suggested by the manufacturer. After transfection, the cells were cultured for 24 h in complete medium and then serum starved for 24 h before cell stimulation, treatment with inhibitors or cell lysis. The vector encoding the HA-tagged forms of Akt (HA-AKT1) and myristoilated Akt (Myr-AKT) were previously described.[30,31] A quick-change site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) and the oligonucleotides listed in Supplementary Table 1 were used to make single amino-acid changes using HA-AKT1 or Myr-AKT plasmids as templates. His6-SUMO1, His6-SUMO2, His6-SUMO3 and SV5-Ubc9 plasmids were previously described.[32,33]

Materials and antibodies

Wortmannin and insulin were purchased from Sigma (Madrid, Spain). Smart-pool small interfering RNAs against Ubc9 (siUbc9) and scramble small interfering RNA (siC) were purchased from Dharmacon (Thermo Scientific, Epson, UK). AKT1 recombinant protein was purchased from Calbiochem (Madrid, Spain). Recombinant p27 was expressed and purified essentially as described.[34] Antibodies to total AKT, phospho-FOXO1/3a (Thr24/32), phospho-P70S6K (Thr-389), phospho-Ser-473 of AKT and phospho-AKT substrate antibody were purchased from Cell Signaling (Izasa SA, Barcelona, Spain). An anti-HA monoclonal antibody was purchased from Covance (Madrid, Spain). Antibodies against PML, FOXO3a and B23 were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-actin antibody was from MP Biomedicals (Illrich, France), anti-tubulin antibody was from Serotec (Düsseldorf, Germany) and anti-GAPDH was from Millipore (Madrid, Spain). Anti-SUMO2/3 antibody was from Invitrogen.

In vitro SUMO conjugation assay

In vitro SUMO conjugation assays were performed on [^{35}S]methionine-labeled *in vitro*-transcribed/translated proteins as described previously.[35]

In vitro deSUMOylation assay

In vitro deSUMOylation assay with GST-SENP1 was performed on AKT1-SUMO1 as described previously.[7]

Immunoprecipitation assay

Cells were lysed in RIPA buffer at 4 °C, centrifuged at 15 800 × *g* for 5 min and immunoprecipitated overnight at 4 °C after addition of 1 µl of the specified antibody and 50 µl of 50% protein A-Sepharose CL-4B beads (GE Healthcare, Madrid, Spain). Beads were then washed four times with RIPA buffer and resuspended in 30 µl of SDS–polyacrylamide gel electrophoresis (PAGE) loading buffer.

AKT *in vitro* kinase assay

Recombinant AKT1 protein was subjected to an *in vitro* SUMOylation assay in the presence or absence of SUMO. Then the result of the SUMOylation assay was employed in an *in vitro* kinase assay using p27 as a substrate. The reaction was stopped by the addition of SDS–PAGE loading buffer, and proteins were separated by SDS–PAGE and transferred to nitrocellulose membrane. Phosphorylated p27 protein was evaluated using antibody anti-p-AKT substrate. The blots were probed with anti-AKT and anti-p27 to quantify protein levels.

Western blot analysis

Cells were washed in phosphate-buffered saline, scraped into SDS–PAGE loading buffer and boiled for 5 min. Proteins of total extracts were separated by SDS–PAGE and transferred onto a nitrocellulose membrane. The membranes were incubated with the primary antibodies. Signals were detected by using chemiluminescence. Quantification of band intensities was performed by using ImageJ software and normalized to total protein densitometry values.

Purification of His-tagged conjugates

The purification of His-tagged conjugates using Ni^{2+}-NTA- agarose beads, allowing the purification of proteins that are covalently conjugated to SUMO, was performed as described previously.[36]

Nucleo-cytoplasm separation

The nuclear and cytoplasmic factions were isolated using a nucleo-cytosol fractionation kit (BioVision), according to manufacturer's protocol.

In vitro growth rate analysis

The *in vitro* growth rates of the cell lines were assessed by MTT proliferation assay.

Focus formation assay

NIH-3T3 cells were co-transfected with pcDNA, Myr-AKT1-WT or Myr-AKT1-SMUT and a puromycin resistance vector at a ratio 10:1. At 48 h after transfection, cells were grown in medium supplemented with puromycin antibiotic for 3 days. At this time, cells were plated in 60-mm dishes and cultured in Dulbecco's modified Eagle's medium containing 5% fetal bovine serum for 14 days, when colonies were visualized by crystal violet staining.

Apoptosis quantification

Apoptosis was quantified by flow cytometry using the caspase-3, active form, mAb Apoptosis kit from BD Pharmigen (BD Biosciences, Madrid, Spain), according to manufacturer's protocol.

Statistical analysis

For statistical analysis between control and different groups, the Student's *t*-test was applied. The significance level chosen for the statistical analysis was $P < 0.05$.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies this paper on the Oncogene website (http://www.nature.com/onc)