Science Signaling
AAAS

` **The Deacetylase SIRT1 Promotes Membrane Localization and Activation of Akt and PDK1 During Tumorigenesis and Cardiac Hypertrophy**

Nagalingam R. Sundaresan, Vinodkumar B. Pillai, Don Wolfgeher, Sadhana Samant, Prabhakaran Vasudevan, Vishwas Parekh, Hariharasundaram Raghuraman, John M. Cunningham, Madhu Gupta and Mahesh P. Gupta (19 July 2011)

Science Signaling **4** (182), ra46. [DOI: 10.1126/scisignal.2001465]

The following resources related to this article are available online at http://stke.sciencemag.org.
This information is current as of 26 September 2011.

Article Tools Visit the online version of this article to access the personalization and article tools:
http://stke.sciencemag.org/cgi/content/full/sigtrans;4/182/ra46

Supplemental Materials *"Supplementary Materials"*
http://stke.sciencemag.org/cgi/content/full/sigtrans;4/182/ra46/DC1

Related Content The editors suggest related resources on *Science*'s sites:
http://stke.sciencemag.org/cgi/content/abstract/sigtrans;2/97/pe76

References This article cites 54 articles, 26 of which can be accessed for free:
http://stke.sciencemag.org/cgi/content/full/sigtrans;4/182/ra46#otherarticles

Glossary Look up definitions for abbreviations and terms found in this article:
http://stke.sciencemag.org/glossary/

Permissions Obtain information about reproducing this article:
http://www.sciencemag.org/about/permissions.dtl

Downloaded from stke.sciencemag.org on September 26, 2011

Science Signaling (ISSN 1937-9145) is published weekly, except the last week in December, by the American Association for the Advancement of Science, 1200 New York Avenue, NW, Washington, DC 20005. Copyright 2008 by the American Association for the Advancement of Science; all rights reserved.

CELL BIOLOGY

The Deacetylase SIRT1 Promotes Membrane Localization and Activation of Akt and PDK1 During Tumorigenesis and Cardiac Hypertrophy

Nagalingam R. Sundaresan,[1] Vinodkumar B. Pillai,[1] Don Wolfgeher,[2] Sadhana Samant,[1] Prabhakaran Vasudevan,[3] Vishwas Parekh,[3] Hariharasundaram Raghuraman,[4] John M. Cunningham,[3] Madhu Gupta,[5] Mahesh P. Gupta[1]*

Signaling through the kinase Akt regulates many biological functions. Akt is activated during growth factor stimulation through a process that requires binding of Akt to phosphatidylinositol 3,4,5-trisphosphate (PIP_3), which promotes membrane localization and phosphorylation of Akt by the upstream kinase PDK1 (phosphoinositide-dependent protein kinase 1). We show that Akt and PDK1 are acetylated at lysine residues in their pleckstrin homology domains, which mediate PIP_3 binding. Acetylation blocked binding of Akt and PDK1 to PIP_3, thereby preventing membrane localization and phosphorylation of Akt. Deacetylation by SIRT1 enhanced binding of Akt and PDK1 to PIP_3 and promoted their activation. Mice injected with cells expressing a mutant that mimicked a constitutively acetylated form of Akt developed smaller tumors than those injected with cells expressing wild-type Akt. Furthermore, impaired Akt activation in the hearts of SIRT1-deficient mice was associated with reduced cardiac hypertrophy in response to physical exercise and angiotensin II. These findings uncover a key posttranslational modification of Akt that is important for its oncogenic and hypertrophic activities.

INTRODUCTION

Akt (also known as protein kinase B) is a serine-threonine kinase that mediates signals involved in regulation of diverse cellular processes, such as cell growth, proliferation, apoptosis, metabolism, and angiogenesis. Aberrations in Akt signaling have been linked with diseases such as cancer, cardiac hypertrophy, diabetes, neuronal degeneration, and vascular disorders (*1*, *2*). Three closely related, highly conserved homologs of Akt are present in mammals, the best-characterized of which is Akt1 (Akt). Akt is generally activated by stimulation of growth factor receptors on the cell surface in a multistep process that includes (i) binding of Akt to phosphatidylinositol 3,4,5-trisphosphate (PIP_3), (ii) translocation of Akt from the cytosol to the membrane, and (iii) phosphorylation of Akt at Thr^{308} and Ser^{473} by the upstream kinases PDK1 (phosphoinositide-dependent protein kinase 1) and mTORC2 [mammalian target of rapamycin (mTOR) complex 2] (*3*–*5*). The initial steps of Akt activation (namely, binding to PIP_3 and membrane translocation) are primarily regulated by the pleckstrin homology (PH) domain, a region present in most of the membrane lipid-binding proteins (*6*). However, the mechanisms that regulate Akt binding to PIP_3 and membrane localization are not yet understood.

Reversible acetylation of lysine residues by histone acetyltransferases (HATs) and histone deacetylases (HDACs) is a posttranslational mechanism that controls the activity of many proteins besides histones, including kinases (*7*, *8*). SIRT1 is a prototypical member of the class III HDACs, collectively called sirtuins, which need NAD to deacetylate substrates (*9*, *10*). SIRT1 has been detected in the nucleus and the cytoplasm (*11*). SIRT1 regulates many of the same cellular processes regulated by Akt (*8*), and SIRT1 activators have been used to treat metabolic disorders characterized by defective Akt signaling (*12*).

Here, we identified reversible acetylation as a potential regulatory mechanism that controls the activity of Akt. Lysine acetylation in the PH domain of Akt inhibited its binding to PIP_3, thereby inactivating Akt. SIRT1 deacetylated the PH domain, a process that was necessary for binding of Akt to PIP_3 and, hence, for its membrane localization and activation. Similarly, SIRT1-dependent deacetylation also promotes PIP_3 binding and membrane localization of PDK1. Using tumor growth and cardiac hypertrophy models, we demonstrated that SIRT1 promotes the development of these diseases through deacetylation-dependent activation of Akt.

RESULTS

Lysine acetylation inhibits Akt activity

Endogenous Akt immunoprecipitated from primary cells (cardiac myocytes and fibroblasts) and from cell lines [HeLa and human embryonic kidney (HEK) 293T] was acetylated (Fig. 1A). Akt was also acetylated in various tissues, including heart, liver, brain, and skeletal muscle (Fig. 1B). Akt is activated by growth factor stimulation or mild cellular stress and is inactivated during cell death. Akt acetylation was reduced in conditions that promote activation of Akt, such as stimulation of cells with insulin-like growth factor 1 (IGF-1) or insulin. In contrast, Akt acetylation was increased in cells treated with apoptosis-inducing agents, such as camptothecin and staurosporine, suggesting that acetylation is inversely related

[1]Department of Surgery, Committee on Cellular and Molecular Physiology, University of Chicago, 5841 South Maryland Avenue, Chicago, IL 60637, USA. [2]Department of Molecular Genetics and Cell Biology, University of Chicago, Chicago, IL 60637, USA. [3]Department of Pediatrics, Committee on Developmental Biology, University of Chicago, Chicago, IL 60637, USA. [4]Department of Biochemistry and Molecular Biology, University of Chicago, Chicago, IL 60637, USA. [5]Department of Physiology and Biophysics, University of Illinois, Chicago, IL 60607, USA.
*To whom correspondence should be addressed. E-mail: mgupta@surgery.bsd.uchicago.edu

Downloaded from stke.sciencemag.org on September 26, 2011

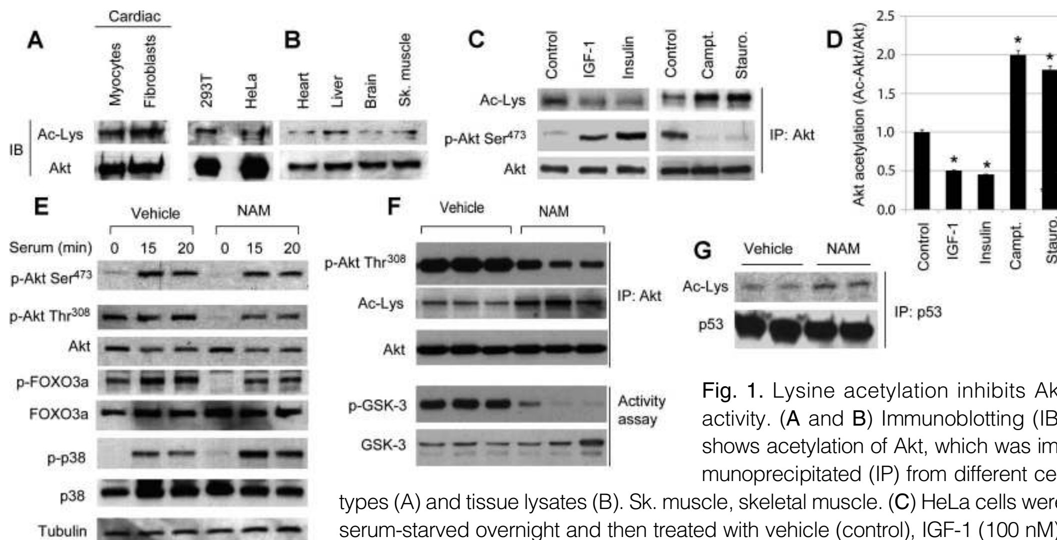

Fig. 1. Lysine acetylation inhibits Akt activity. (A and B) Immunoblotting (IB) shows acetylation of Akt, which was immunoprecipitated (IP) from different cell types (A) and tissue lysates (B). Sk. muscle, skeletal muscle. (C) HeLa cells were serum-starved overnight and then treated with vehicle (control), IGF-1 (100 nM), or insulin (100 nM) for 15 min (left). HeLa cells were grown in complete growth medium and treated with camptothecin (Campt; 10 μM) or staurosporine (Stauro; 1 μM) for 2 hours (right). Akt immunoprecipitates were immunoblotted with the indicated antibodies. (D) Quantification of Akt acetylation in HeLa cells treated with vehicle (control), IGF-1 (100 nM), or insulin (100 nM) for 15 min or treated with camptothecin (10 μM) or staurosporine for 2 hours. Values are means ± SE. $n = 5$ experiments (*$P < 0.01$). (E) COS-7 cells were serum-starved and treated with NAM overnight and then stimulated with serum for different time periods. Whole-cell lysates (WCLs) were immunoblotted with the indicated antibodies. (F) The phosphorylation status and acetylation status of Akt immunoprecipitated from HEK293T cells treated with NAM were determined by immunoblotting. Akt activity was assessed in kinase assays with GSK-3 peptide as substrate. The results shown are from an experiment performed in triplicate. (G) The acetylation status of p53 immunoprecipitated from cell lysates in (F) was determined by immunoblotting. Ac, acetylation; P, phosphorylation; 293T, HEK293T cells; Sk. muscle, skeletal muscle. Blots are representative of $n = 3$ experiments.

to Akt activity (Fig. 1, C and D). Cells treated with the class III HDAC inhibitor nicotinamide (NAM) showed increased Akt acetylation, a finding that was associated with reduced phosphorylation of Akt at Thr^{308} and Ser^{473}, reduced phosphorylation of the substrate FOXO3a, and reduced kinase activity [as assessed by phosphorylation of a glycogen synthase kinase 3 (GSK-3) peptide] in vitro. However, phosphorylation of p38 (which is not a substrate for Akt) was unaltered (Fig. 1, E to G). These data demonstrated that acetylation inhibits Akt phosphorylation and that this posttranslational modification is sensitive to stress and growth stimuli that influence Akt activity.

Akt is acetylated at Lys^{14} and Lys^{20} in the PH domain

Two widely distributed mammalian HATs, p300 and PCAF, bind to and acetylate Akt in vitro as well as in cells (Fig. 2, A to C). Overexpression of PCAF (p300/CBP-associated factor) or p300 in HEK293T cells reduced phosphorylation of Akt, consistent with reduced activity of Akt toward GSK-3 peptide (Fig. 2D). Furthermore, Akt activity was increased in lysates prepared from HEK293T cells depleted of PCAF by small interfering RNA (siRNA) or from HeLa cells depleted of p300 by siRNA (Fig. 2, E to H). Thus, both HATs can acetylate Akt in cells, a process that inhibits Akt activity.

To delineate the acetylated region of Akt, we analyzed different deletion mutants of Akt. Full-length Akt, but not a mutant lacking the PH domain, was acetylated (Fig. 3A). To identify acetylated Lys residues of Akt, we subjected immunoprecipitated Akt from HeLa cells to mass spectrometry (MS) and found that Akt was acetylated at Lys^{14} and Lys^{20} in the PH domain (fig. S1). The relative stoichiometries of Lys^{14} and Lys^{20} acetylation were 53.5% and 50.2%, respectively (table S1). Substitution of Lys residues with Arg conserves the net positive charge of the amino acid but prevents neutralization of charge by acetylation, whereas substitution of Lys with Gln resembles the acetylated Lys in terms of charge. Forms of Akt with Lys^{14}→Arg (K14R) or Lys^{14}→Gln (K14Q) mutations showed reduced phosphorylation in cells (Fig. 3B). In contrast, an Akt form with a Lys^{20}→Arg (K20R) mutation showed increased phosphorylation, whereas an Akt form with a Lys^{20}→Gln (K20Q) mutation showed reduced phosphorylation (Fig. 3B). Additionally, activity assays showed that the K20Q mutant, but not the K20R mutant, had higher activity toward GSK-3 peptide, whereas the K14R and K14Q mutants were nearly completely inert (Fig. 3C). These data indicated that acetylation-mediated regulation of Akt activity is likely to be controlled by modification of Lys^{14} and Lys^{20}. Lys^{14} is involved in the binding of the phosphate groups of PIP_3 to Akt (*13*), and mutation of this Lys will therefore eliminate binding of Akt to PIP_3. Additionally, posttranslational modification of Lys^{14} might also impair direct binding of Akt to PIP_3.

SIRT1 deacetylates and activates Akt

Our results from NAM-treated cells indicated that a member of the sirtuin family might be the deacetylase that targets Akt. Akt immunoprecipitates from HEK293T cells contained SIRT1, and conversely, SIRT1 coimmunoprecipitated with Akt (Fig. 4, A and B). Furthermore, this interaction occurred preferentially when cells were stimulated with serum (fig. S2, A and B). Glutathione *S*-transferase (GST) pull-down assays using different deletion mutants of Akt revealed that SIRT1 bound to the PH domain of Akt, the same region that contains the acetylated Lys residues (fig. S3). SIRT1 coimmunoprecipitated with full-length Akt but not with a mutant lacking PH domain (Fig. 4C). Furthermore, expression of SIRT1 increased phosphorylation at Thr^{308} and Ser^{473} sites in full-length Akt but not in the mutant lacking the PH domain (Fig. 4C), thus demonstrating that SIRT1 binds to and activates Akt through its PH domain. In vitro assays indicated that SIRT1 deacetylated the PH domain of Akt in a NAD-dependent manner (Fig. 4D). Acetylation of Akt was higher in cells expressing PCAF alone than those coexpressing PCAF and SIRT1 (fig. S4A). Expression of wild-type SIRT1, but not a catalytically inactive mutant (His^{335}→Ala; H335A), increased phosphorylation of Akt and its substrate FOXO3a in cells and activity of Akt, findings that were associated with deacetylation of Akt, indicating that SIRT1-mediated deacetylation activates Akt (Fig. 4E).

We asked whether Lys^{20} is needed for SIRT1-mediated activation of Akt. The phosphorylation status of Akt was determined in cells coexpressing

Downloaded from stke.sciencemag.org on September 26, 2011

SIRT1 with wild-type Akt, the K20R mutant, or the K20Q mutant. SIRT1 increased phosphorylation of wild-type Akt but not that of K20R or K20Q mutants, suggesting that deacetylation of Lys^{20} is necessary for SIRT1-mediated activation of Akt (Fig. 4F). To test whether endogenous SIRT1 could deacetylate and activate Akt, we used HeLa cells maintained in low- or high-glucose medium or treated with resveratrol, a small-molecule activator of SIRT1 (*14*). Compared to cells grown in high-glucose (25 mM) medium, phosphorylation of Akt and its targets was increased in cells grown in low-glucose (1 mM) medium, conditions that should activate SIRT1. Similarly, resveratrol treatment, which increases SIRT1 abundance, increased phosphorylation of Akt and its substrates (fig. S4, B to D).

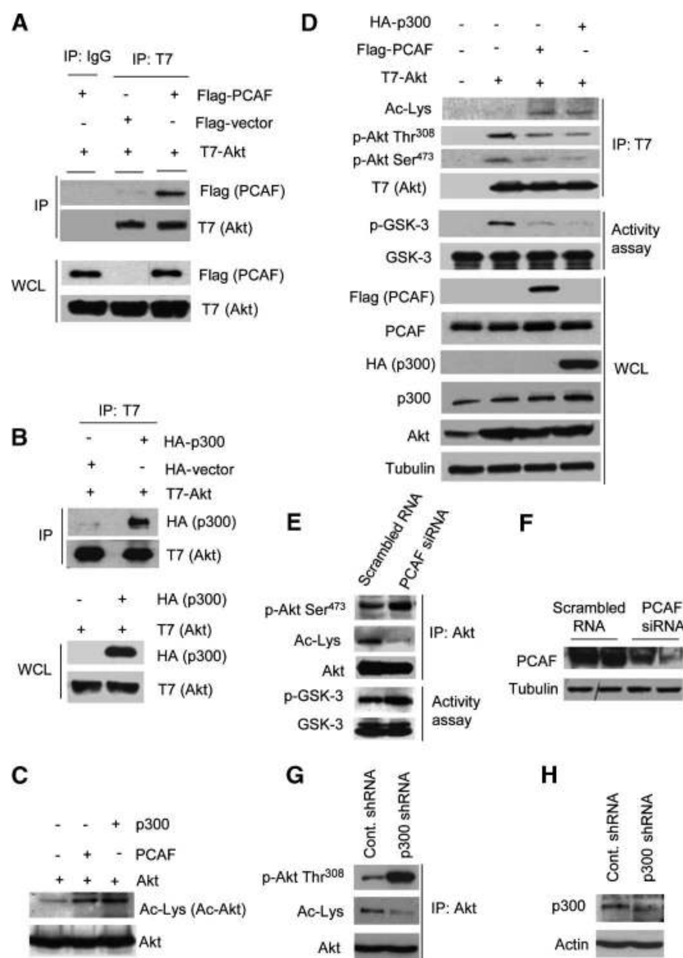

Fig. 2. PCAF and p300 acetylate Akt. (A and B) Akt immunoprecipitates from transfected HeLa cells were immunoblotted with the indicated antibodies. (C) Immunoblot showing in vitro acetylation of Akt by PCAF or p300. (D) Akt immunoprecipitates from transfected HEK293T cells were analyzed by immunoblotting with the indicated antibodies (top) and in kinase assays with GSK-3 peptide as substrate (middle). WCLs were immunoblotted with the indicated antibodies (bottom). (E) Akt immunoprecipitates from HEK293T cells transfected with siRNAs directed against PCAF immunoblotted with the indicated antibodies. Akt activity was determined in kinase assays with GSK-3 peptide as substrate. (F) Immunoblot showing PCAF knockdown in HEK293T cells. (G) Akt immunoprecipitates from HeLa cells expressing a short hairpin RNA (shRNA) directed against p300 were immunoblotted with the indicated antibodies. (H) Immunoblot showing p300 knockdown in HeLa cells. Blots are representative of n = 2 experiments.

To test whether SIRT1 activates Akt in vivo under physiological conditions, we fasted mice overnight to activate SIRT1 and then injected them with insulin. Skeletal muscle lysate prepared from fasted mice showed increased SIRT1 activation and insulin-mediated Akt activation compared to lysate from control mice fed ad libitum (Fig. 5A). In addition, phosphorylation of FOXO3a was greater in fasted mice treated with insulin compared to controls, and FOXO-dependent gene expression was activated in SIRT1-deficient cells (fig. S5, A to C), consistent with a previous report (*15*). Moreover, SIRT1-deficient mouse embryo fibroblasts (MEFs) showed reduced IGF-1–mediated activation of Akt compared to wild-type MEFs (Fig. 5B). This phenotype was rescued by expression of SIRT1 (fig. S6, A and B). Thus, under conditions that promote cellular growth, endogenous SIRT1 deacetylates and activates Akt.

We asked next whether deacetylation is a necessary step for phosphorylation of Akt. A time course analysis of Akt acetylation and phosphorylation in serum-stimulated HeLa cells showed that deacetylation of Akt occurred before phosphorylation (Fig. 5C). Surprisingly, in cells treated with the phosphatidylinositol 3-kinase (PI3K) inhibitor GDC0941, which blocks Akt phosphorylation, no deacetylation of Akt could be detected (Fig. 5C), suggesting that PI3K also plays a role in the events controlling Akt deacetylation. SIRT1 is activated by increases in the ratio of the oxidized (NAD) and reduced (NADH) forms of cellular nicotinamide adenine dinucleotide (*9*). In addition to inducing deacetylation of Akt, serum stimulation increased the cellular NAD/NADH ratio, suggesting that deacetylation of Akt under growth conditions may be mediated by increased activity of SIRT1 (fig. S7).

Lysine acetylation regulates the binding of Akt to PIP_3

A critical step in the process of Akt activation is the binding of Akt to PIP_3 through its PH domain, a process that facilitates Akt membrane localization (*16*, *17*). Because acetylation of the PH domain inhibited Akt activity, we hypothesized that acetylation might also regulate the binding of PH domain to PIP_3. In a lipid-protein overlay assay, deacetylated but not acetylated Akt bound to PIP_3 in a concentration-dependent manner (Fig. 6A). However, deacetylation did not change the affinity of Akt to other lipids (fig. S8A), thus suggesting a specific effect of acetylation on the binding ability of Akt to PIP_3. Acetylated Akt-PH domain bound less to PIP_3 compared to its nonacetylated counterpart (fig. S8B). Akt from SIRT1-expressing cells bound to PIP_3-conjugated beads more efficiently than did Akt from cells expressing the SIRT1 H355A mutant (Fig. 6B). Similarly, PIP_3 beads pulled down more Akt from wild-type cells than from SIRT1-deficient cells, suggesting that SIRT1-mediated deacetylation facilitated Akt binding to PIP_3 (Fig. 6C). PI3K activity was not significantly different in wild-type and SIRT1-deficient cells, thus excluding the possibility that cellular PIP_3 abundance differed between these cells (fig. S8C), suggesting that SIRT1 does not influence the synthesis of PIP_3. The K20R mutant, which mimics deacetylated Akt, readily bound to PIP_3, whereas the K20Q mutant, which mimics constitutively acetylated Akt, did not bind to PIP_3, and mutating the Lys^{14} to either Arg or Gln rendered Akt incapable of binding to PIP_3 (Fig. 6D). Thus, reversible acetylation of Lys^{20} in the PH domain regulates the binding of Akt to PIP_3.

SIRT1 promotes membrane localization of Akt

Because SIRT1-mediated deacetylation enhanced Akt binding to PIP_3, we reasoned that SIRT1 might also play a role in membrane localization of Akt. IGF-1 treatment increased the amount of Akt in the membrane fraction of wild-type but not SIRT1 knockout (SIRT1-KO) MEFs, suggesting

Downloaded from stke.sciencemag.org on September 26, 2011

that SIRT1 promoted IGF-1–induced membrane localization of Akt (Fig. 7A). Furthermore, IGF-1 treatment promoted membrane localization of the green fluorescent protein (GFP)–Akt–PH fusion protein in wild-type MEFs but not in SIRT1-KO cells (Fig. 7B). We next asked whether deacetylation of Lys^{14} and Lys^{20} in the PH domain influenced the membrane localization of Akt. In serum-stimulated HeLa cells, GFP-Akt-PH fusion proteins with K14Q or K14R mutations did not localize to the plasma membrane before or after serum stimulation, whereas the membrane localization of the GFP-Akt-PH construct with the K20Q mutation was reduced compared to that of the construct with the K20R mutation, which mimics the deacetylated Lys^{20} form of Akt (Fig. 7C). Together, these results indicate that reversible acetylation of Lys^{20} in the PH domain regulates membrane localization of Akt.

Some human cancers have a mutation in the PH domain of Akt (Glu^{17}→Lys; E17K) that confers constitutive activation of Akt (*18*). Ubiquitination of this E17K mutant promotes membrane localization and activation of Akt (*19*). A form of the GFP-Akt-PH construct with the E17K mutation was primarily membrane-localized, whereas a form of this construct with K17K/K20Q double mutations was not membrane-localized (Fig. 7D), thus indicating that besides ubiquitination (*19*), deacetylation of the PH domain can also play a critical role in the membrane localization of Akt.

Lysine acetylation of the PH domain regulates the membrane localization and activation of PDK1

PDK1 is an upstream activating kinase of Akt, which must also bind to PIP_3 for membrane localization and activation (*20*). Because PDK1 also contains a PH domain, we hypothesized that like Akt, membrane localization of PDK1 might also be regulated by reversible acetylation. PCAF could acetylate PDK1 in vitro (Fig. 8A), and tandem MS (MS/MS) analysis identified two acetylated lysine residues, Lys^{495} and Lys^{534}, in the PH domain of PDK1 (fig. S9). SIRT1 bound to PDK1 in HeLa cells and deacetylated PDK1 in vitro (Fig. 8, A and B). In prostate cancer PC3 cells stimulated with IGF-1, SIRT1 expression increased phosphorylation of PDK1 at Ser^{241} in the membrane fraction but not in the cytoplasmic fraction (Fig. 8C). We also noticed that a large amount of SIRT1 was present not only in the cytoplasmic fraction, as expected, but also in the membrane fraction. Imaging analysis indicated that under conditions expected to promote growth, endogenous SIRT1 and exogenously

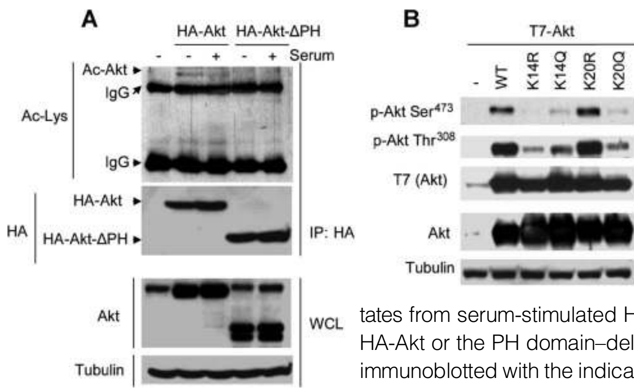

Fig. 3. Akt is acetylated at Lys^{14} and Lys^{20} in the PH domain. (**A**) Akt immunoprecipitates from serum-stimulated HEK293T cells expressing full-length HA-Akt or the PH domain–deleted Akt mutant (HA-Akt-ΔPH) were immunoblotted with the indicated antibodies. WCLs were immunoblotted with the indicated antibodies. (**B**) The phosphorylation status of Akt immunoprecipitated from cells expressing wild-type (WT) T7-Akt or T7-Akt mutants was determined by immunoblotting (upper panels). Immunoblots of WCLs (bottom panel). (**C**) Activity of WT T7-Akt or T7-Akt mutants was determined in kinase assays with GSK-3 peptide as substrate. Blots are representative of n = 4 experiments.

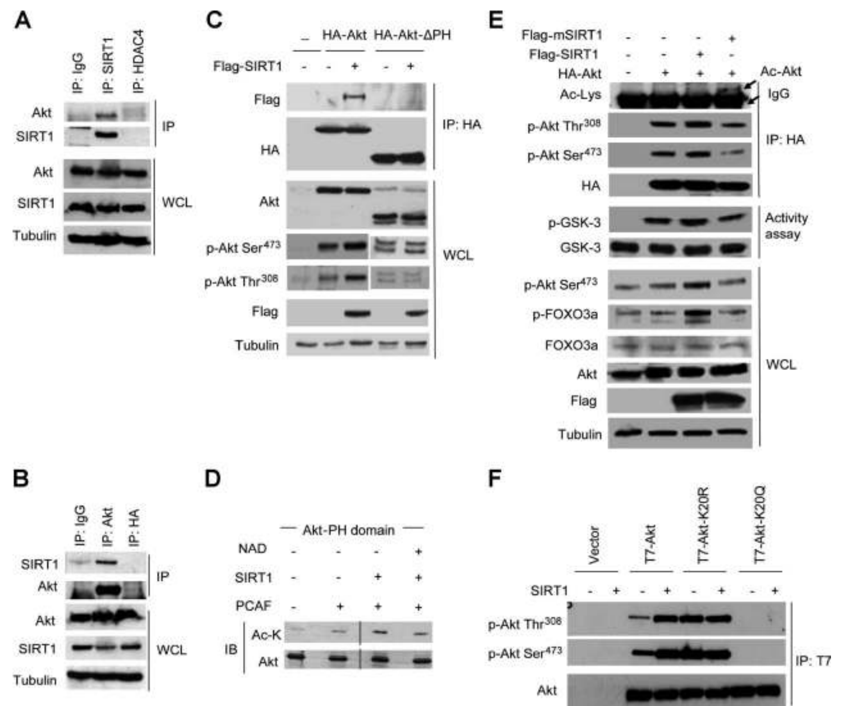

Fig. 4. SIRT1-mediated deacetylation activates Akt. (**A**) Endogenous Akt coprecipitates with SIRT1, but not with HDAC4, from HEK293T cells. (**B**) SIRT1 coprecipitates with Akt but not with HA tag. (**C**) SIRT1 interacts with the PH domain of Akt. Akt immunoprecipitates from cells expressing Flag. SIRT1 and full-length Akt or the PH domain–deleted Akt mutant were immunoblotted for coprecipitation of SIRT1. The doublets produced by the mutants lacking the PH domain are likely a result of the use of a cryptic methionine residue. (**D**) Immunoblot showing SIRT1-dependent deacetylation of the Akt-PH domain in vitro. (**E**) The phosphorylation status and acetylation status of Akt immunoprecipitated from cells expressing Akt and WT or mutant SIRT1 were determined by immunoblot. The activity of Akt was determined with kinase assays using GSK-3 peptide as substrate. WCLs were immunoblotted with the indicated antibodies. IgG, immunoglobulin G. (**F**) Deacetylation of Lys^{20} is necessary for Akt activation. Akt immunoprecipitates from HEK293T cells expressing different acetylation-deficient mutants of Akt in combination with SIRT1 were immunoblotted with the indicated antibodies. Blots are representative of n = 2 experiments.

Downloaded from stke.sciencemag.org on September 26, 2011

expressed SIRT1 were localized at the plasma membrane (Fig. 8D and fig. S10, A to C).

Binding of PDK1 to PIP_3 was higher in cells expressing wild-type SIRT1 compared to cells expressing the catalytically inactive SIRT1 mutant, suggesting that SIRT1 enhances the ability of PDK1 to bind to PIP_3 (Fig. 8, E and F). In serum-starved PC3 cells treated with IGF-1, PDK1 phosphorylation and membrane localization were reduced in cells lacking SIRT1, compared to cells with SIRT1, at all time points of IGF-1 stimulation examined (Fig. 8G and fig. S11). Thus, these data demonstrate that, like Akt, SIRT1 deacetylates and activates PDK1.

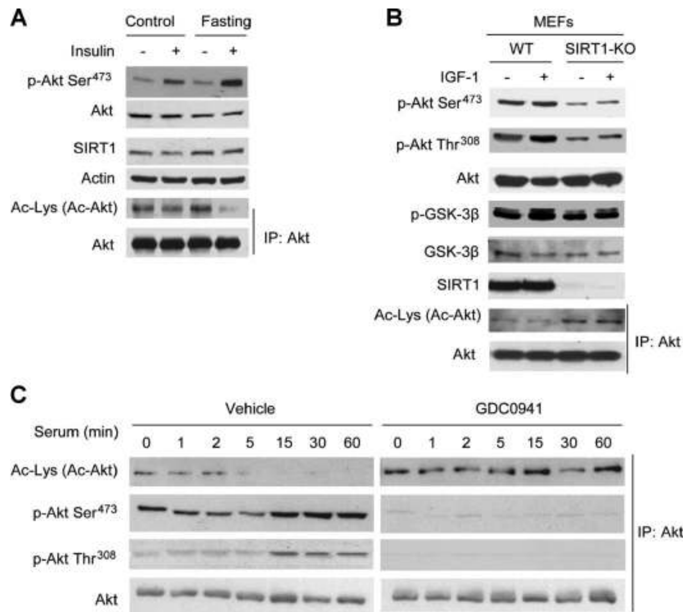

Fig. 5. Endogenous SIRT1 regulates growth factor–mediated Akt activation. (**A**) Mice fed ad libitum (control) or fasted overnight were injected with insulin or vehicle intraperitoneally. Muscle tissue was harvested 30 min after insulin injection and immunoblotted for the indicated proteins. The acetylation status of immunoprecipitated Akt was determined by immunoblotting. (**B**) IGF-1–treated MEFs from WT and SIRT1-KO mice were immunoblotted with the indicated antibodies. (**C**) Deacetylation of Akt occurs before phosphorylation. HeLa cells were serum-starved overnight, treated with vehicle or the PI3K inhibitor GDC0941, and stimulated with serum for different time periods. The acetylation status and phosphorylation status of immunoprecipitated Akt were determined by immunoblotting with the indicated antibodies. Blots are representative of *n* = 2 experiments.

SIRT1 controls Akt-mediated cell survival and tumorigenesis

To understand the functional role of SIRT1-mediated activation of Akt, we analyzed the effect of depleting endogenous SIRT1 or overexpression of SIRT1 on the survival and proliferation capacity of PC3 cells, processes that are Akt-dependent (*19*). SIRT1 deficiency caused reduced Akt phosphorylation and significantly increased cell death (Fig. 9, A and B, and fig. S12), whereas overexpression of SIRT1 promoted cell proliferation (Fig. 9, C and D). To test whether deacetylation of the Akt-PH domain induces tumor-permissive phenotypes, we developed stable HeLa cells expressing T7 tag alone, T7-Akt, T7-Akt-K20R, or T7-Akt-K20Q mutants. T7-Akt– and T7-Akt-K20R–expressing cells formed stress fibers, showed cytoplasmic or perinuclear localization of β-catenin (or both) and increased abundance of smooth muscle α-actin, characteristics of a more aggressive tumor phenotype not seen in T7-Akt-K20Q–expressing cells (Fig. 9, E and F). Stable HeLa cells expressing T7 tag alone, T7-Akt, or T7-Akt-K20Q were injected into nude mice. After 8 weeks, large tumors were detected in mice injected with cells expressing T7-Akt (wild-type cells) but not in mice injected with cells expressing Akt-K20Q (Fig. 9G and fig. S13), suggesting that Akt acetylation inhibits its tumorigenic activity. We could not assess the tumorigenic activity of Akt-K20R mutant because these cells lost stable expression of Akt-K20R after multiple passages.

SIRT1-KO mice show a cardiac phenotype reminiscent of Akt deficiency

We also assessed the cardiac phenotype of SIRT1-KO and SIRT1-overexpressing transgenic mice. Like Akt-deficient mice, SIRT1-KO mice die neonatally, and the few surviving mice show reduced heart weight–to–tibia length (HW/TL) ratio and reduced cardiac myocyte size, phenotypes that suggest defective physiologic cardiac growth (Fig. 10A and fig. S14, A and B). SIRT1-KO hearts also showed reduced phosphorylation of Akt and GSK-3β compared to wild-type control hearts (Fig. 10B). Similar results were found in liver lysates (fig. S15). Akt immunoprecipitated from lysates of SIRT1-KO hearts showed increased acetylation, reduced phosphorylation, and reduced activity toward GSK-3 peptide compared to that immunoprecipitated from wild-type heart lysate (Fig. 10, C and D), thus suggesting that SIRT1 promotes Akt activity in the heart.

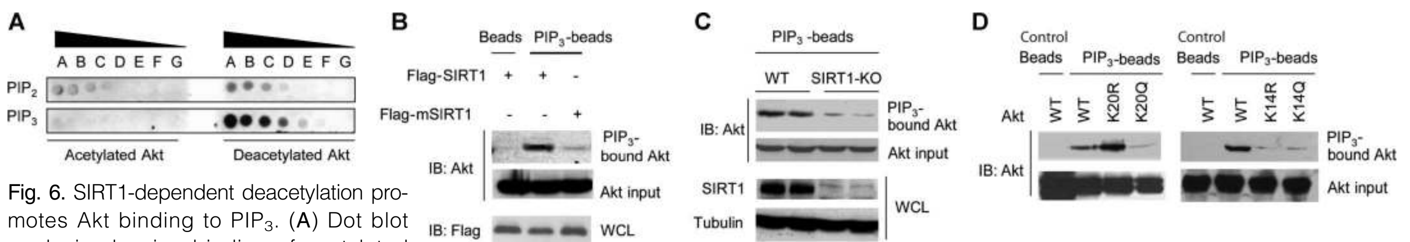

Fig. 6. SIRT1-dependent deacetylation promotes Akt binding to PIP_3. (**A**) Dot blot analysis showing binding of acetylated and deacetylated Akt to PIP_3 and PIP_2. Dots A to G are decreasing amounts of lipids, ranging from 100 to 1.56 pmol per spot. PIP_2, phosphatidylinositol 4,5-bisphosphate. (**B**) Lysates of HeLa cells expressing WT or mutant SIRT1 were incubated with PIP_3-conjugated beads. Binding of Akt to beads was detected by immunoblotting. (**C**) Immunoblot showing binding of PIP_3-conjugated beads to Akt from WT or SIRT1-KO liver lysates. (**D**) Immunoblot showing binding of WT, K20R, or K20Q Akt to PIP_3. Blots are representative of *n* = 4 experiments.

Downloaded from stke.sciencemag.org on September 26, 2011

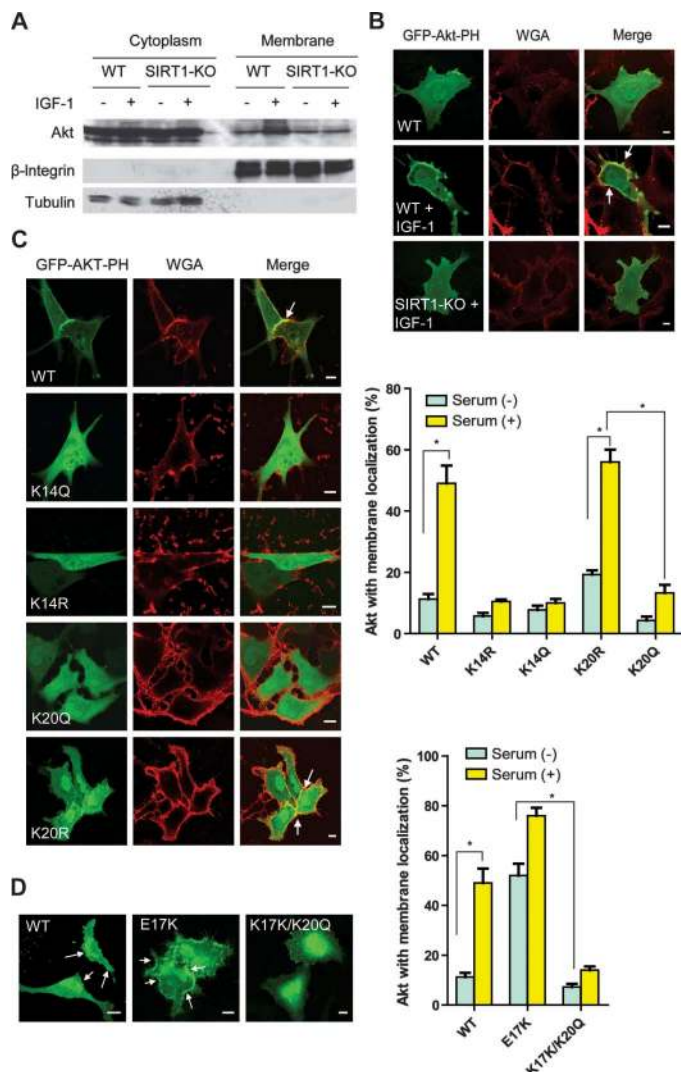

Fig. 7. SIRT1-mediated deacetylation promotes membrane localization of Akt. (A) Immunoblot showing IGF-1–induced localization of Akt to the membrane fraction of WT but not of SIRT1-KO MEFs. The cytoplasmic and membrane fraction markers tubulin and β-integrin were also immunoblotted. $n = 2$ experiments. (B) WT and SIRT1-KO MEFs expressing GFP-Akt-PH domain were stimulated with IGF-1 for 15 min. WGA is a membrane marker. The membrane localization of GFP-tagged protein (arrows) was examined by confocal microscopy. Scale bars, 10 μm. (C and D) HeLa cells expressing GFP-Akt-PH domain constructs carrying different mutations were stimulated with serum for 15 min, and the membrane localization of GFP protein (arrows) was determined by confocal microscopy. Images are representative of $n = 3$ experiments. Scale bars, 10 μm [(C) and (D)]. Quantification of data in (C) and (D) shown in bar graphs. Cells (100 to 200) were scored, and representative results are from $n = 3$ experiments (mean ± SE, $*P < 0.001$).

Akt activation has been implicated in the development of cardiac hypertrophy (*21*, *22*). In response to a hypertrophic stimulus, angiotensin II (AngII), SIRT1-KO mice failed to develop cardiac hypertrophy, whereas wild-type mice showed a hypertrophic response as well as induction of the genes encoding ANP (atrial natriuretic peptide), BNP (brain natriuretic peptide), and β-MHC (β–myosin heavy chain) (Fig. 10A and figs. S14 and S16). To test whether defects in Akt signaling might contribute to the lack of hypertrophic response of SIRT1-KO hearts, we examined phosphorylation of Akt and of its downstream targets. Compared to wild-type hearts, SIRT1-KO hearts had reduced phosphorylation of Akt in response to agonist treatment, which was associated with reduced phosphorylation of downstream targets of Akt, such as GSK-3β, mTOR, and FOXO3a, but not p38, which is not an Akt target (Fig. 10E). We also analyzed the development of exercise-induced physiological hypertrophy in wild-type and SIRT1-KO mice. Whereas wild-type mice developed ~20% cardiac hypertrophy after 8 weeks of forced swim exercise, SIRT1-KO mice were resistant to this type of hypertrophy (fig. S17A). Furthermore, control cardiomyocytes with SIRT1, but not SIRT1-deficient cardiomyocytes, developed robust hypertrophy in response to IGF-1, suggesting that SIRT1 is needed for IGF-1–mediated hypertrophy of cardiomyocytes (fig. S17, B and C). In addition, the hearts of transgenic mice overexpressing SIRT1 (at about fourfold higher abundance over that of the endogenous protein) developed significant cardiac hypertrophy under basal conditions, showed increased phosphorylation and deacetylation of Akt, and increased the activity of Akt toward its substrates (Fig. 10, F and G). To confirm that SIRT1-mediated cardiac hypertrophy requires Akt activation, we treated SIRT1-overexpressing cardiomyocytes with the Akt inhibitor triciribine. SIRT1-mediated hypertrophy of cardiomyocytes was blocked by inhibiting Akt (fig. S18), thus suggesting a functional interaction between these two proteins. Akt is activated in a pressure-overload model of cardiac hypertrophy induced by transverse aortic constriction (TAC) (*23*). SIRT1 was activated in mice that underwent TAC, and hypertrophic responses, as well as activation of Akt and SIRT1, were further enhanced by treatment of these mice with resveratrol and blocked by treating mice with triciribine (Fig. 10, H and I). These results thus demonstrate that SIRT1 activation is needed for pro-hypertrophic activity of Akt in the heart.

DISCUSSION

Akt and SIRT1 regulate many common biological functions, and in the heart, SIRT1 deficiency results in a phenotype reminiscent of Akt deficiency. We have found that during growth factor stimulation of cells, SIRT1 binds to and activates Akt by enhancing its binding to PIP_3 and thereby increasing membrane localization and phosphorylation of Akt. We identified two conserved lysine residues in the PH domain of Akt, which are subject to modification by acetylation. SIRT1-dependent deacetylation of these residues was necessary for binding of Akt to PIP_3. These findings disclosed a new step in Akt signaling.

Akt activity is regulated by reversible acetylation of the PH domain

Acetylation of the PH domain inactivated Akt because of its inability to bind to PIP_3. This effect could be resulting from acetylation-induced disruption of the tertiary structure of the PH domain of Akt, which is necessary for its binding to PIP_3. The PH domain contains a bipolar structure in which one side of the domain is a positively charged surface composed of basic amino acids, whereas the other side is populated by acidic residues, which form the ligand-binding pocket in its center (*6*). We suggest that acetylation, which neutralizes the positive charge of lysine, could affect the bipolar structure of the PH domain of Akt. This explanation is supported by the observation that mutation of Lys^{14} or Lys^{20} residues to glutamine rendered Akt incapable of binding to PIP_3,

Downloaded from stke.sciencemag.org on September 26, 2011

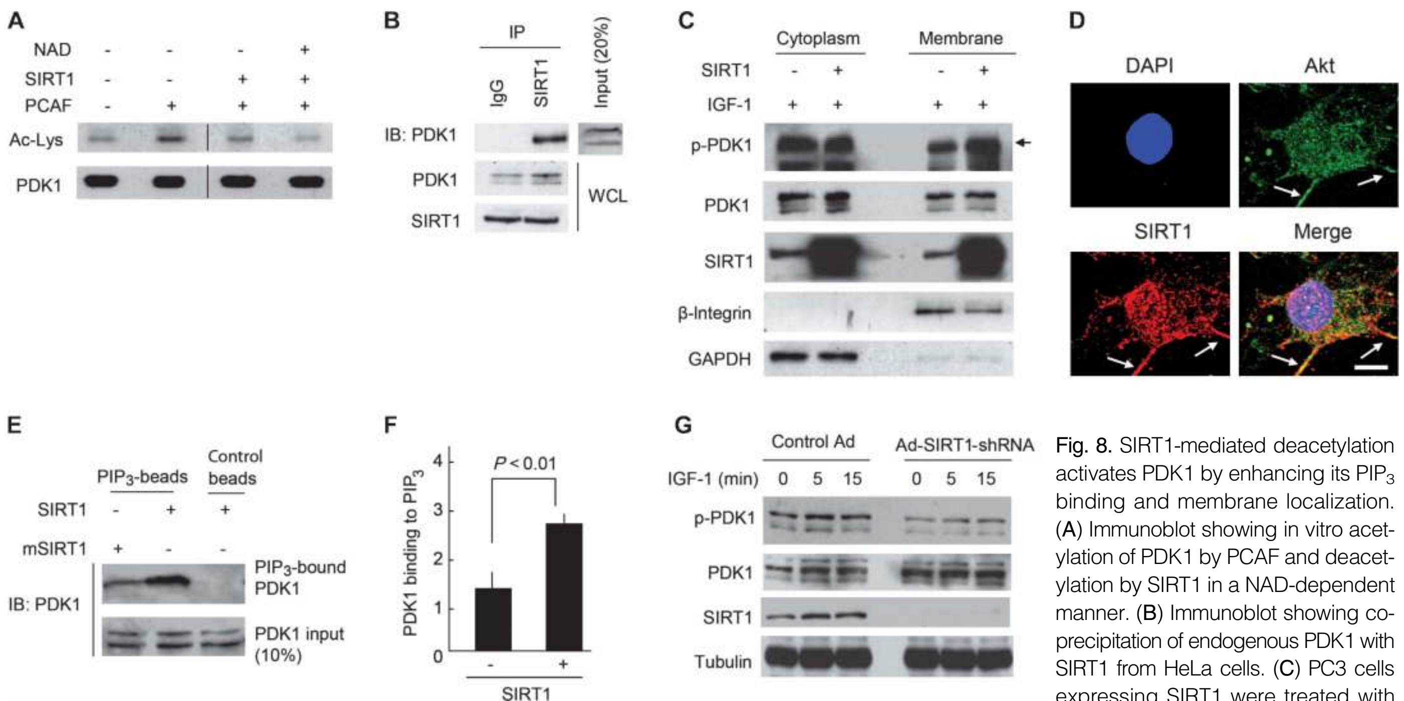

Fig. 8. SIRT1-mediated deacetylation activates PDK1 by enhancing its PIP_3 binding and membrane localization. (**A**) Immunoblot showing in vitro acetylation of PDK1 by PCAF and deacetylation by SIRT1 in a NAD-dependent manner. (**B**) Immunoblot showing coprecipitation of endogenous PDK1 with SIRT1 from HeLa cells. (**C**) PC3 cells expressing SIRT1 were treated with IGF-1. Cytoplasmic and membrane fractions were prepared and immunoblotted for the indicated proteins. Phosphorylation of PDK1 at Ser^{241} was increased in the membrane fraction of SIRT1-expressing cells after IGF-1 treatment (arrow). Blots are representative of $n = 2$ experiments. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. (**D**) Cardiac fibroblasts were stimulated with serum and then immunostained for SIRT1 and Akt. Membrane localization of proteins was examined by confocal imaging. Arrows denote colocalization of Akt and SIRT1 at the membrane. Images are representative of $n = 3$ experiments. Scale bar, 20 μm. (**E**) Lysates of HeLa cells expressing WT or mutant SIRT1 were incubated with PIP_3-conjugated beads. Binding of PDK1 to beads was detected by immunoblotting. (**F**) Quantification of PDK1-PIP_3 binding. Values are means ± SE. $n = 3$ experiments. (**G**) PC3 cells infected with adenovirus (Ad) encoding shRNA directed against SIRT1 were serum-starved overnight and then treated with IGF-1 for the indicated time points. WCLs were immunoblotted for the indicated proteins. Blots are representative of $n = 2$ experiments.

suggesting that electrostatic interactions are crucial for the binding of PIP_3 to these residues and thus the PH domain. Nearly all PH domains have a similar arrangement of positively charged Lys residues, which are essential for their binding to PIP_3. Our data showing that acetylation of the PH domain of PDK1 at Lys^{495}, which is homologous to Lys^{14} of Akt, rendered PDK1 inactive suggest that acetylation of PH domains could be a common mechanism that inhibits the activity of PIP_3 binding proteins.

In addition to acetylation, ubiquitination can promote Akt membrane localization and activation. Yang *et al.* have reported that the E3 ubiquitin ligase TRAF6 mediates ubiquitination of Akt at Lys^8 and Lys^{14}, thereby promoting translocation of Akt to the plasma membrane upon exposure to IGF-1. Mutation of Lys^8 to Arg impairs translocation of Akt to the membrane but has no effect on the binding of Akt to PIP_3, suggesting that membrane localization and PIP_3 binding of Akt are two independent events (*19*). Similarly, in our study, defects in membrane localization of the K20Q mutant might have resulted from impaired binding to PIP_3, rather than defects in their translocation to the membrane, because the ubiquitination sites (Lys^8 and Lys^{14}) of these mutants were intact. Another possibility is that deacetylation of Lys^{20} and Lys^{14}, the latter of which is also ubiquitinated (*19*), facilitates ubiquitination-mediated transport of Akt to the membrane. Figure 11 presents a model explaining how lysine acetylation and ubiquitination could regulate Akt membrane localization, PIP_3 binding, and activation.

SIRT1 deacetylates and activates Akt under growth conditions

We identified SIRT1 as a HDAC that can bind to and deacetylate Akt and that promotes increased membrane localization of Akt during growth factor signaling. However, in the absence of growth factor stimulation, we did not find Akt binding to SIRT1, suggesting that Akt and SIRT1 may not interact with each other under basal conditions and that growth factor signaling is needed for their interaction (fig. S2, A and B). How growth factor signaling promotes their interaction is not known at this point and warrants further investigation. Consistent with our observation, Akt has been reported to activate SIRT1 by destabilizing miR-199a, which targets SIRT1, thus suggesting that there could be a positive feedback loop in which these proteins regulate each other's activity under certain specified conditions (*24*, *25*).

Implications of the SIRT1-Akt interaction in promoting longevity

Both Akt and SIRT1 have been implicated in life-span determination of many organisms. Akt is a downstream component of the insulin–IGF-1 signaling pathway, mutations in which reduce the activity of the IGF-PI3K-Akt signaling pathway and extend the life spans of *Caenorhabditis elegans*, *Drosophila*, and mice (*26*). Inhibition of Akt also extends the life span of human endothelial cells (*27*). This effect of Akt inhibition has been attributed to increased FOXO-dependent transcription of the

Downloaded from stke.sciencemag.org on September 26, 2011

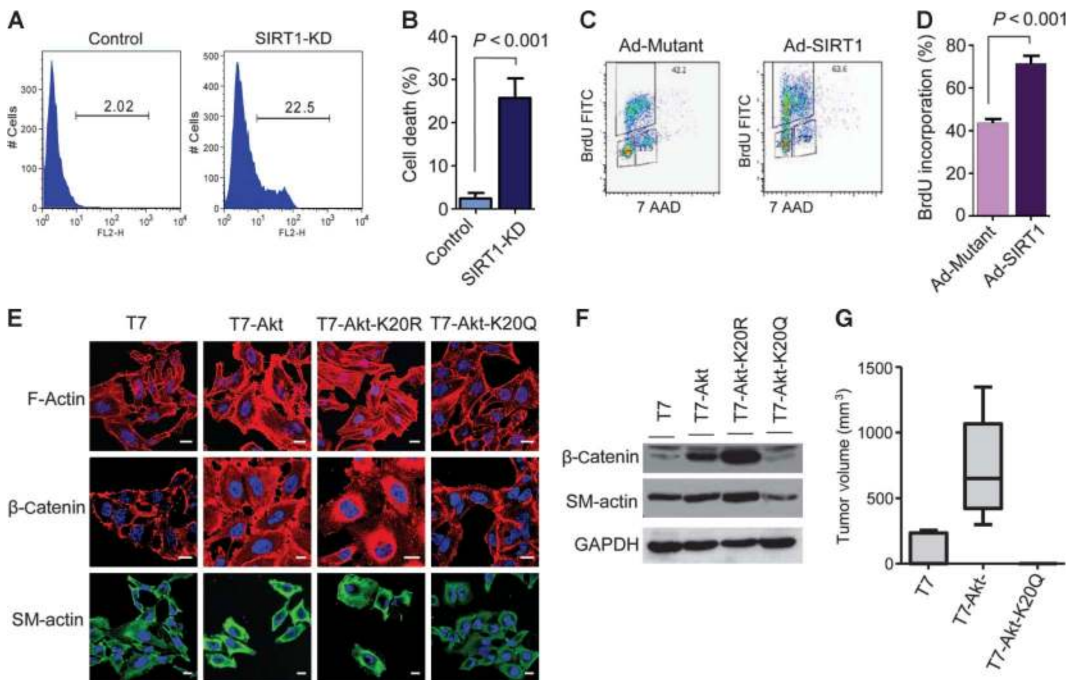

Fig. 9. SIRT1 regulates Akt-mediated cell survival and tumorigenesis. (A) Cell death of PC3 cells was determined 72 hours after knockdown (KD) of SIRT1. (B) Quantification of cell death. Values are means ± SE. n = 3 sets of cells. (C) Cell proliferation in PC3 cells overexpressing SIRT1 was monitored by incorporation of BrdU. FITC, fluorescein isothiocyanate. (D) Quantification of BrdU incorporation in cells. Mean ± SE. n = 4 sets of cells. (E) Stable HeLa cells expressing the indicated proteins were immunostained for smooth muscle α-actin (SM-actin) and β-catenin. F-actin was visualized with rhodamine-phalloidin. Scale bars, 20 μm. n = 3 sets of cells. (F) Immunoblot of cells from (E). (G) Stable HeLa cells expressing indicated protein were injected into nude mice, and tumor growth was monitored after 8 weeks. Values are means ± SE. n = 6 mice per group.

gene encoding superoxide dismutase, an enzyme that helps to reduce cellular oxidative stress. Although Akt activation decreases life-span extension, there is a lack of consensus regarding the role of SIRT1 in this process. Activation of SIRT1 homologs can increase the life span of yeast, *C. elegans*, and *Drosophila* (*28*); however, this concept has been challenged by others who found no correlation between life-span extension and activation of SIRT1 (*29*–*31*). Overexpression of SIRT1 does not extend the replicative life span of human fibroblasts or prostate epithelial cells, but it causes replicative senescence in response to chronic stress (*32*, *33*). Here, we found that SIRT1-mediated deacetylation can enhance Akt activity upon growth factor stimulation and not in the absence of growth factor signaling. Consistent with our results, SIRT1 has been reported to be necessary for IGF-1 signaling, and inhibition of SIRT1 protects neurons from oxidative stress–mediated damage (*29*). Thus, it is likely that under growth-stimulating conditions (such as when insulin signaling is active), SIRT1 activates Akt, resulting in phosphorylation and inactivation of FOXO, because of its export from the nucleus. In contrast, in the absence of insulin signaling, lack of SIRT1-mediated activation of Akt facilitates nuclear localization of FOXO, where it promotes expression of genes involved in stress resistance, endurance, and longevity. Consistent with this notion, SIRT1 interacts with FOXO3a in serum-deprived cells and not under basal conditions when growth factors are present (*34*). Thus, it appears that SIRT1 may promote longevity under caloric restriction but not in the presence of growth factors. This dual activity of SIRT1 under different conditions could account for the lack of consensus regarding its role in promoting longevity.

SIRT1-Akt signaling is involved in cancer and cardiac hypertrophy

Akt is considered a central player in tumorigenesis (*35*). Increased activation of Akt in cancer cells occurs through several PI3K-dependent and PI3K-independent mechanisms (*18*). Ubiquitination, which promotes recruitment of Akt to the membrane, enhances the oncogenic activity of Akt (*19*). Our data demonstrate that SIRT1, which facilitates localization of Akt to the membrane, also promotes the oncogenic activity of Akt. The role of SIRT1 in cancer is complex and not well understood (*36*). Induction of SIRT1 by caloric restriction reduces cell proliferation and tumor formation in a mouse model of colon cancer (*37*). These observations differ from other reports in which SIRT1 abundance is increased in various types of tumors, including prostate, leukemia, colon, and skin (*36*). These tumors also show increased activation of Akt, suggesting that SIRT1 might promote cancer by activating Akt (*35*, *38*).

Both Akt and SIRT1 play a critical role in the development of cardiac hypertrophy and angiogenesis (*23*, *39*, *40*). Akt-deficient mice are resistant to swimming-induced cardiac hypertrophy, suggesting a role for Akt in physiological growth of the heart (*41*). In contrast, mice with cardiac-specific overexpression of Akt develop cardiac hypertrophy under basal conditions (*42*). Both Akt and SIRT1-KO mice die neonatally, and the few surviving mice exhibit reduced HW/TL ratio compared to their wild-type littermates. Here, hearts of SIRT1-null mice showed impaired cardiac hypertrophic response, whereas those of SIRT1-overexpressing mice showed robust cardiac hypertrophy under basal conditions. The similar cardiac phenotypes between Akt and SIRT1-KO mice and mice overexpressing these proteins may result from the ability of SIRT1 to control the activity of Akt in the heart. Consistent with our results, spontaneously hypertensive rats with left ventricular hypertrophy show increased cardiac mRNA abundance for SIRT1 (*43*). Furthermore, mice with a liver-specific ablation of SIRT1 show reduced Akt phosphorylation despite having increased blood insulin concentrations (*44*). Similarly, overexpression of SIRT1 increases Akt phosphorylation in senescent endothelial cells (*45*). Also, transgenic mice with gain of function of SIRT1 have been reported to be protected from diabetes and metabolic disorders, which may result from enhanced Akt signaling (*44*, *46*).

In summary, we have demonstrated that SIRT1-dependent deacetylation of the PH domain is necessary for the activation of Akt under growth conditions and possibly of other PH domain proteins. These findings are

Downloaded from stke.sciencemag.org on September 26, 2011

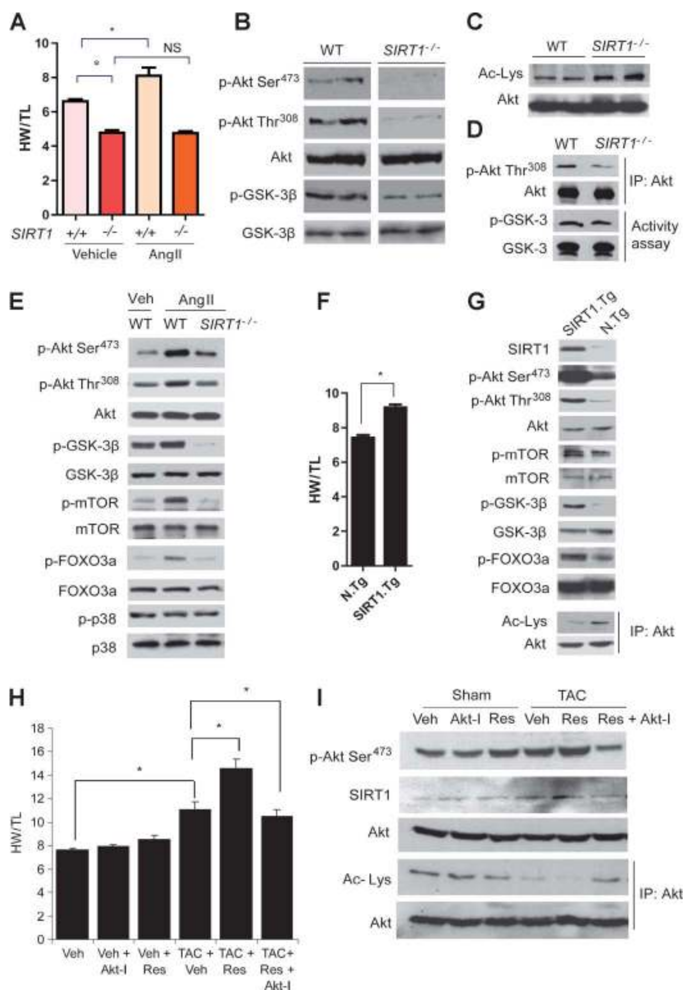

Fig. 10. SIRT1-KO mice exhibit Akt-defective phenotype in the heart. (A) Ratio of heart weight (HW) to tibia length (TL) of WT and SIRT1-KO adult mice treated with AngII to induce cardiac hypertrophy. Values are means ± SE. $n = 5$ mice per group. (B) Immunoblot showing abundance of different proteins in heart lysates of WT and SIRT1-KO mice. (C and D) The acetylation status (C) and phosphorylation status (D) of Akt immunoprecipitated from heart lysates of WT and SIRT1-KO mice were determined by immunoblotting. Akt activity was assessed in kinase assay with GSK-3 peptide as substrate. (E) Immunoblot of cardiac lysates from WT and SIRT1-KO mice treated with AngII to induce hypertrophy. (F) HW/TL ratio of 6-week-old N.Tg (nontransgenic) and SIRT1.Tg mice with cardiac-specific expression of SIRT1. Values are means ± SE. $n = 5$ mice per group ($^*P < 0.05$). (G) Immunoblot showing phosphorylation of Akt and its substrates, GSK-3β (Ser^9), mTOR (Ser^{2448}), and FOXO3a (Thr^{32}) in hearts from N.Tg and SIRT1.Tg mice (top). Acetylation status of Akt (bottom). (H) Akt inhibition attenuates cardiac hypertrophy mediated by activation of endogenous SIRT1. Mice fed a standard chow or diet containing resveratrol (Res) were subjected to TAC for 8 weeks. Akt inhibitor (Akt-I), triciribine (1.5 mg/kg), or vehicle (Veh) was injected daily to block endogenous Akt activity. After 2 weeks, mice were killed and the HW/TL ratio was measured. Values are means ± SE. $n = 6$ mice per group ($^*P < 0.05$). (I) Immunoblot of heart lysates showing Akt phosphorylation and SIRT1 activation. Bottom: Acetylation status of Akt in different groups of hearts. Blots are representative of $n = 2$ experiments.

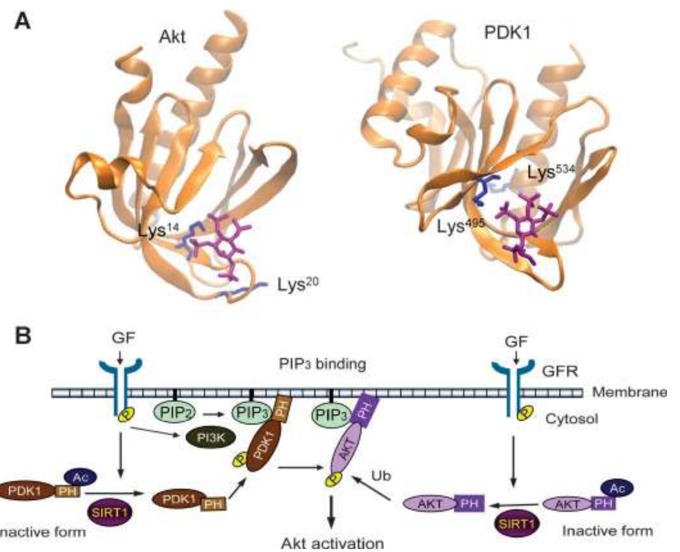

Fig. 11. (A) Crystal structures of PH domains of Akt and PDK1 interacting with PIP_3. The acetylated lysines are shown in blue, and PIP_3 is shown in purple (*54*). Lys^{14} (Akt) and Lys^{495} (PDK1) are in the binding pocket of the PH domain and are involved in direct binding to PIP_3. In Akt, Lys^{20} is present in the variable loop and is in close proximity to the binding pocket in inactive or apo form. Lys^{20} undergoes considerable conformational change upon binding with PIP_3 (bound form, Protein Data Bank code 1UNQ). Neutralization of the charge on Lys^{20} might hinder the binding of PIP_3 and the conformational changes associated with this binding event, thereby leading to inactivation of Akt. (B) A model illustrating the role of lysine acetylation in regulating the membrane localization and activation of Akt. The PH domains of Akt and PDK1 are acetylated, leading to inhibition of their binding to PIP_3 and hence inactivation. During growth factor (GF) stimulation of cells, SIRT1 deacetylates Akt as well as PDK1, enabling them to bind to PIP_3, which is generated from PIP_2 by activation of PI3K during GF receptor stimulation. PIP_3 binding promotes localization of Akt and PDK1 to the plasma membrane. It is also possible that deacetylation makes lysine residue (or residues) available for ubiquitination (Ub), thereby facilitating ubiquitination-mediated transport of proteins to the plasma membrane. PDK1 phosphorylates Akt at the plasma membrane, leading to activation of Akt.

important for understanding the interrelationship between kinases and deacetylases in regulating cell signaling.

MATERIALS AND METHODS

Mice, cell cultures, and reagents

SIRT1-KO mice were provided by M. W. McBurney. The transgenic mice with cardiac-specific overexpression of SIRT1 and methods to prepare cardiomyocyte cultures from 2-day-old neonatal rat hearts have been described earlier (*47*). For all adenovirus infections, viruses were used at a multiplicity of infection (MOI) of 100. HeLa and HEK293T cells were grown in Dulbecco's modified Eagle's medium, and PC3 cells were grown in RPMI 1640 supplemented with penicillin-streptomycin and 10% fetal bovine serum. MEFs were prepared using standard protocols. Experiments with MEFs were performed from passage 2 to 4. T7-Akt1, Flag-PCAF,

Downloaded from stke.sciencemag.org on September 26, 2011

hemagglutinin (HA)–tagged p300, Flag-SIRT1, Flag-mSIRT1 (H355A), and GFP-Akt-PH constructs were obtained from Addgene. T7-Akt1 and GFP-Akt-PH constructs were used to generate K14R, K14Q, K20R, K20Q, K17E, and E17K/K20Q mutants with a site-directed mutagenesis kit from Stratagene Inc. pCMV-HA mammalian expression vector (Clontech) was used to generate HA-tagged Akt1 or ΔPH-Akt1. IGF-1 and insulin were purchased from Sigma. Recombinant SIRT1 protein was purchased from Abcam. Recombinant PCAF and p300 proteins were purchased from Millipore. Antibodies were purchased from Cell Signaling, Millipore, Sigma, and Santa Cruz Biotechnology. Triciribine was purchased from Calbiochem Inc., and GDC0941 was bought from Chemdea Inc. For in vivo experiments, triciribine was used at a dose of 1.5 mg/kg intraperitoneally (*48*). Stable HeLa cells expressing T7 tag alone, T7-Akt, T7-Akt-K20R, or T7-Akt-K20Q were generated by means of standard protocols. The concentration of G418 used for stable selection was 500 μg/ml. SIRT1 and its catalytic mutant (H355A) overexpressing adenoviruses were described previously (*49*).

Cell death and proliferation assays

Apoptosis of PC3 cells was analyzed by fluorescence-activated cell sorting (FACS) by means of the annexin V–phycoerythrin apoptosis detection kit (BD Pharmingen). Cell proliferation was determined by quantifying 5-bromo-2′-deoxyuridine (BrdU) incorporation with a commercial kit (BD Pharmingen).

Subcellular fractionation, immunoprecipitation, and immunoblot analyses

Cytoplasmic and membrane fractions were prepared with a ProteoExtract Subcellular Proteome Extraction Kit (Calbiochem). Immunoblotting and immunoprecipitation experiments were done using standard protocols as described previously (*50*).

In vitro acetylation-deacetylation assay

Akt1 bound to beads was incubated with 200 ng of active PCAF or p300, 0.5 mM acetyl coenzyme A (CoA) (Sigma), 50 mM NAM, and 10 μM trichostatin A (TSA) in HAT buffer [50 mM tris (pH 8.0), 10% glycerol, 0.1 mM EDTA, and 1 mM dithiothreitol] for 30 min at 30°C on a rotator. Nonacetylated Akt1 contained all these components except acetyl CoA. Reactions were terminated by the addition of SDS sample buffer. For the deacetylation assay, in vitro acetylated Akt1 bound to beads was resuspended in a HDAC buffer [25 mM tris-HCl (pH 8.0), 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, and bovine serum albumin (0.1 mg/ml)] with or without 500 μM NAD. SIRT1 (50 ng/μl) was added to the reaction mixture, and it was incubated for 1 hour at 30°C on a nutator. Proteins were resolved by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by Western blotting with an anti–Ac-Lys antibody (*51*).

Akt activity assay

Endogenous Akt was immunoprecipitated with agarose beads conjugated with either T7, HA, or specific Akt antibody. The activity of Akt was determined with an Akt assay kit and GSK-3 peptide (which contains the Ser^9 and Ser^{21} phosphorylation sites of GSK-3α and GSK-3β) as a substrate (Cell Signaling).

PIP_3 binding assay

Lysates from HeLa cells expressing T7-Akt or the different T7-Akt mutants were incubated with control or PIP_3-conjugated agarose beads (Echelon). Akt bound to PIP_3 was detected by immunoblotting with an antibody against T7 tag (Novagen) or Akt. For in vitro protein lipid overlay assay, HeLa cells expressing HA-tagged Akt were then treated with TSA (10 μM) and NAM (50 mM) to induce acetylation of Akt endogenously. Akt protein was immunoprecipitated with HA-affinity agarose beads and subjected to in vitro deacetylation reactions with recombinant SIRT1 in the presence or absence of NAD. After completion of the reaction, beads were washed, and acetylated and deacetylated proteins were eluted from the beads with a HA-peptide column. PIP_3 and other membrane lipid–coated strips were purchased from Echelon Biosciences. Binding of acetylated and deacetylated Akt to membrane lipids was analyzed according to the manufacturer's instructions.

In vivo tumorigenesis

HeLa cells stably expressing constructs expressing T7 tag, T7-Akt1, T7-Akt-K20Q, or T7-Akt-K20R were selected with G418 (500 μg/ml), and at the 30th passage, 2×10^6 cells were subcutaneously injected into 6-week-old athymic nude mice. Tumor growth was monitored for 8 weeks. Tumor size was measured with a caliper, and tumor volume was determined with a standard formula, $L \times W^2 \times 0.52$, where L is the longest diameter and W is the shortest diameter. T7-Akt-K20R–expressing clones were not injected into mice, because these clones lost their stable expression of the construct after multiple passages.

Induction of hypertrophy in mice

All animal protocols were reviewed and approved by the University of Chicago Institutional Animal Care and Use Committee. AngII was delivered chronically at a rate of 3.0 mg/kg per day for 14 days by implanting osmotic mini pumps (ALZET model 2002) in the peritoneal cavity of mice. Control mice underwent the same procedure except that their pumps were filled with vehicle. For experiments with resveratrol, mice were fed a diet containing resveratrol (0.067%) for 4 weeks before induction of pressure overload and were maintained on the same diet throughout the course of the study (*47*). Pressure overload hypertrophy was induced by TAC of ascending aorta of mice, as described elsewhere (*47*). The measurement of hypertrophy of cardiomyocytes and the expression of hypertrophic marker genes were carried out essentially as described earlier (*52*, *53*).

Statistical analysis

All values are expressed as means ± SE. Statistical differences among groups were determined with either Student's t test (for two groups) or one-way analysis of variance (ANOVA) with Bonferroni's post test (for more than two groups) by use of GraphPad Prism Software.

SUPPLEMENTARY MATERIALS

www.sciencesignaling.org/cgi/content/full/4/182/ra46/DC1
Fig. S1. Akt is acetylated at Lys^{14} and Lys^{20} in the PH domain.
Fig. S2. SIRT1 binds to Akt under growth-promoting conditions.
Fig. S3. SIRT1 binds to the PH domain of Akt.
Fig. S4. SIRT1 deacetylates and activates Akt in cells.
Fig. S5. SIRT1-depleted cells show increased FOXO transcriptional activity.
Fig. S6. SIRT1 overexpression rescues phosphorylation of Akt in SIRT1-KO cells.
Fig. S7. Growth factor treatment of cells increases the NAD/NADH ratio.
Fig. S8. Acetylation specifically alters the binding ability of Akt to PIP_3.
Fig. S9. The PH domain of PDK1 is acetylated at Lys^{495} and Lys^{534}.
Fig. S10. SIRT1 localizes to the plasma membrane under growth-promoting conditions.
Fig. S11. SIRT1-deficient PC3 cells do not show IGF-1–induced phosphorylation of PDK1.
Fig. S12. SIRT1-deficient PC3 cells show reduced activation of Akt.
Fig. S13. Lys^{20} acetylation inhibits the tumorigenic activity of Akt.
Fig. S14. *SIRT1*$^{-/-}$ mice show reduced AngII-mediated cardiac hypertrophy.
Fig. S15. *SIRT1*$^{-/-}$ mouse liver shows reduced Akt phosphorylation.
Fig. S16. AngII infusion fails to induce hypertrophic markers in *SIRT1*$^{-/-}$ mice.
Fig. S17. SIRT1 deficiency blocks the development of physiologic hypertrophy.
Fig. S18. The Akt inhibitor triciribine blocks SIRT1-mediated hypertrophy of cardiomyocytes.
Table S1. Relative stoichiometry of acetylation of the PH domain of Akt.

Downloaded from stke.sciencemag.org on September 26, 2011

REFERENCES AND NOTES

1. T. F. Franke, D. R. Kaplan, L. C. Cantley, PI3K: Downstream AKTion blocks apoptosis. *Cell* **88**, 435–437 (1997).
2. B. D. Manning, L. C. Cantley, AKT/PKB signaling: Navigating downstream. *Cell* **129**, 1261–1274 (2007).
3. D. R. Alessi, S. R. James, C. P. Downes, A. B. Holmes, P. R. Gaffney, C. B. Reese, P. Cohen, Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Bα. *Curr. Biol.* **7**, 261–269 (1997).
4. A. D. Kohn, F. Takeuchi, R. A. Roth, Akt, a pleckstrin homology domain containing kinase, is activated primarily by phosphorylation. *J. Biol. Chem.* **271**, 21920–21926 (1996).
5. D. D. Sarbassov, D. A. Guertin, S. M. Ali, D. M. Sabatini, Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science* **307**, 1098–1101 (2005).
6. M. J. Rebecchi, S. Scarlata, Pleckstrin homology domains: A common fold with diverse functions. *Annu. Rev. Biophys. Biomol. Struct.* **27**, 503–528 (1998).
7. C. Choudhary, C. Kumar, F. Gnad, M. L. Nielsen, M. Rehman, T. C. Walther, J. V. Olsen, M. Mann, Lysine acetylation targets protein complexes and co-regulates major cellular functions. *Science* **325**, 834–840 (2009).
8. T. Finkel, C. X. Deng, R. Mostoslavsky, Recent progress in the biology and physiology of sirtuins. *Nature* **460**, 587–591 (2009).
9. L. Bordone, L. Guarente, Calorie restriction, SIRT1 and metabolism: Understanding longevity. *Nat. Rev. Mol. Cell Biol.* **6**, 298–305 (2005).
10. S. Michan, D. Sinclair, Sirtuins in mammals: Insights into their biological function. *Biochem. J.* **404**, 1–13 (2007).
11. M. Tanno, J. Sakamoto, T. Miura, K. Shimamoto, Y. Horio, Nucleocytoplasmic shuttling of the NAD$^+$-dependent histone deacetylase SIRT1. *J. Biol. Chem.* **282**, 6823–6832 (2007).
12. J. C. Milne, P. D. Lambert, S. Schenk, D. P. Carney, J. J. Smith, D. J. Gagne, L. Jin, O. Boss, R. B. Perni, C. B. Vu, J. E. Bemis, R. Xie, J. S. Disch, P. Y. Ng, J. J. Nunes, A. V. Lynch, H. Yang, H. Galonek, K. Israelian, W. Choy, A. Iffland, S. Lavu, O. Medvedik, D. A. Sinclair, J. M. Olefsky, M. R. Jirousek, P. J. Elliott, C. H. Westphal, Small molecule activators of SIRT1 as therapeutics for the treatment of type 2 diabetes. *Nature* **450**, 712–716 (2007).
13. C. C. Thomas, M. Deak, D. R. Alessi, D. M. van Aalten, High-resolution structure of the pleckstrin homology domain of protein kinase B/Akt bound to phosphatidylinositol (3,4,5)-trisphosphate. *Curr. Biol.* **12**, 1256–1262 (2002).
14. M. T. Borra, B. C. Smith, J. M. Denu, Mechanism of human SIRT1 activation by resveratrol. *J. Biol. Chem.* **280**, 17187–17195 (2005).
15. M. C. Motta, N. Divecha, M. Lemieux, C. Kamel, D. Chen, W. Gu, Y. Bultsma, M. McBurney, L. Guarente, Mammalian SIRT1 represses forkhead transcription factors. *Cell* **116**, 551–563 (2004).
16. L. Stephens, K. Anderson, D. Stokoe, H. Erdjument-Bromage, G. F. Painter, A. B. Holmes, P. R. Gaffney, C. B. Reese, F. McCormick, P. Tempst, J. Coadwell, P. T. Hawkins, Protein kinase B kinases that mediate phosphatidylinositol 3,4,5-trisphosphate–dependent activation of protein kinase B. *Science* **279**, 710–714 (1998).
17. D. Stokoe, L. R. Stephens, T. Copeland, P. R. Gaffney, C. B. Reese, G. F. Painter, A. B. Holmes, F. McCormick, P. T. Hawkins, Dual role of phosphatidylinositol-3,4,5-trisphosphate in the activation of protein kinase B. *Science* **277**, 567–570 (1997).
18. J. D. Carpten, A. L. Faber, C. Horn, G. P. Donoho, S. L. Briggs, C. M. Robbins, G. Hostetter, S. Boguslawski, T. Y. Moses, S. Savage, M. Uhlik, A. Lin, J. Du, Y. W. Qian, D. J. Zeckner, G. Tucker-Kellogg, J. Touchman, K. Patel, S. Mousses, M. Bittner, R. Schevitz, M. H. Lai, K. L. Blanchard, J. E. Thomas, A transforming mutation in the pleckstrin homology domain of AKT1 in cancer. *Nature* **448**, 439–444 (2007).
19. W. L. Yang, J. Wang, C. H. Chan, S. W. Lee, A. D. Campos, B. Lamothe, L. Hur, B. C. Grabiner, X. Lin, B. G. Darnay, H. K. Lin, The E3 ligase TRAF6 regulates Akt ubiquitination and activation. *Science* **325**, 1134–1138 (2009).
20. K. E. Anderson, J. Coadwell, L. R. Stephens, P. T. Hawkins, Translocation of PDK-1 to the plasma membrane is important in allowing PDK-1 to activate protein kinase B. *Curr. Biol.* **8**, 684–691 (1998).
21. G. Condorelli, A. Drusco, G. Stassi, A. Bellacosa, R. Roncarati, G. Iaccarino, M. A. Russo, Y. Gu, N. Dalton, C. Chung, M. V. Latronico, C. Napoli, J. Sadoshima, C. M. Croce, J. Ross Jr., Akt induces enhanced myocardial contractility and cell size in vivo in transgenic mice. *Proc. Natl. Acad. Sci. U.S.A.* **99**, 12333–12338 (2002).
22. I. Shiojima, M. Yefremashvili, Z. Luo, Y. Kureishi, A. Takahashi, J. Tao, A. Rosenzweig, C. R. Kahn, E. D. Abel, K. Walsh, Akt signaling mediates postnatal heart growth in response to insulin and nutritional status. *J. Biol. Chem.* **277**, 37670–37677 (2002).
23. K. Walsh, Akt signaling and growth of the heart. *Circulation* **113**, 2032–2034 (2006).
24. S. Rane, M. He, D. Sayed, L. Yan, D. Vatner, M. Abdellatif, An antagonism between the AKT and β-adrenergic signaling pathways mediated through their reciprocal effects on miR-199a-5p. *Cell. Signal.* **22**, 1054–1062 (2010).
25. S. Rane, M. He, D. Sayed, H. Vashistha, A. Malhotra, J. Sadoshima, D. E. Vatner, S. F. Vatner, M. Abdellatif, Downregulation of miR-199a derepresses hypoxia-inducible factor-1α and Sirtuin 1 and recapitulates hypoxia preconditioning in cardiac myocytes. *Circ. Res.* **104**, 879–886 (2009).
26. J. A. M. J. L. Janssen, S. W. J. Lamberts, IGF-I and longevity. *Horm. Res.* **62**, 104–109 (2004).
27. H. Miyauchi, T. Minamino, K. Tateno, T. Kunieda, H. Toko, I. Komuro, Akt negatively regulates the in vitro lifespan of human endothelial cells via a p53/p21-dependent pathway. *EMBO J.* **23**, 212–220 (2004).
28. J. A. Baur, D. A. Sinclair, Therapeutic potential of resveratrol: The in vivo evidence. *Nat. Rev. Drug Discov.* **5**, 493–506 (2006).
29. Y. Li, W. Xu, M. W. McBurney, V. D. Longo, SirT1 inhibition reduces IGF-I/IRS-2/Ras/ERK1/2 signaling and protects neurons. *Cell Metab.* **8**, 38–48 (2008).
30. M. Kaeberlein, The ongoing saga of sirtuins and aging. *Cell Metab.* **8**, 4–5 (2008).
31. S. R. Narala, R. C. Allsopp, T. B. Wells, G. Zhang, P. Prasad, M. J. Coussens, D. J. Rossi, I. L. Weissman, H. Vaziri, SIRT1 acts as a nutrient-sensitive growth suppressor and its loss is associated with increased AMPK and telomerase activity. *Mol. Biol. Cell* **19**, 1210–1219 (2008).
32. K. F. Chua, R. Mostoslavsky, D. B. Lombard, W. W. Pang, S. Saito, S. Franco, D. Kaushal, H. L. Cheng, M. R. Fischer, N. Stokes, M. M. Murphy, E. Appella, F. W. Alt, Mammalian SIRT1 limits replicative life span in response to chronic genotoxic stress. *Cell Metab.* **2**, 67–76 (2005).
33. E. Michishita, J. Y. Park, J. M. Burneskis, J. C. Barrett, I. Horikawa, Evolutionarily conserved and nonconserved cellular localizations and functions of human SIRT proteins. *Mol. Biol. Cell* **16**, 4623–4635 (2005).
34. A. Brunet, L. B. Sweeney, J. F. Sturgill, K. F. Chua, P. L. Greer, Y. Lin, H. Tran, S. E. Ross, R. Mostoslavsky, H. Y. Cohen, L. S. Hu, H. L. Cheng, M. P. Jedrychowski, S. P. Gygi, D. A. Sinclair, F. W. Alt, M. E. Greenberg, Stress-dependent regulation of FOXO transcription factors by the SIRT1 deacetylase. *Science* **303**, 2011–2015 (2004).
35. J. R. Testa, A. Bellacosa, AKT plays a central role in tumorigenesis. *Proc. Natl. Acad. Sci. U.S.A.* **98**, 10983–10985 (2001).
36. C. X. Deng, SIRT1, is it a tumor promoter or tumor suppressor? *Int. J. Biol. Sci.* **5**, 147–152 (2009).
37. R. Firestein, G. Blander, S. Michan, P. Oberdoerffer, S. Ogino, J. Campbell, A. Bhimavarapu, S. Luikenhuis, R. de Cabo, C. Fuchs, W. C. Hahn, L. P. Guarente, D. A. Sinclair, The SIRT1 deacetylase suppresses intestinal tumorigenesis and colon cancer growth. *PLoS One* **3**, e2020 (2008).
38. B. T. Hennessy, D. L. Smith, P. T. Ram, Y. Lu, G. B. Mills, Exploiting the PI3K/AKT pathway for cancer drug discovery. *Nat. Rev. Drug Discov.* **4**, 988–1004 (2005).
39. R. R. Alcendor, S. Gao, P. Zhai, D. Zablocki, E. Holle, X. Yu, B. Tian, T. Wagner, S. F. Vatner, J. Sadoshima, Sirt1 regulates aging and resistance to oxidative stress in the heart. *Circ. Res.* **100**, 1512–1521 (2007).
40. M. Potente, L. Ghaeni, D. Baldessari, R. Mostoslavsky, L. Rossig, F. Dequiedt, J. Haendeler, M. Mione, E. Dejana, F. W. Alt, A. M. Zeiher, S. Dimmeler, SIRT1 controls endothelial angiogenic functions during vascular growth. *Genes Dev.* **21**, 2644–2658 (2007).
41. B. DeBosch, I. Treskov, T. S. Lupu, C. Weinheimer, A. Kovacs, M. Courtois, A. J. Muslin, Akt1 is required for physiological cardiac growth. *Circulation* **113**, 2097–2104 (2006).
42. T. Matsui, L. Li, J. C. Wu, S. A. Cook, T. Nagoshi, M. H. Picard, R. Liao, A. Rosenzweig, Phenotypic spectrum caused by transgenic overexpression of activated Akt in the heart. *J. Biol. Chem.* **277**, 22896–22901 (2002).
43. L. Li, L. Zhao, W. Yi-Ming, Y.-S. Yu, C.-Y. Xia, J.-L. Duan, D.-F. Su, Sirt1 hyperexpression in SHR heart related to left ventricular hypertrophy. *Can. J. Physiol. Pharmacol.* **87**, 56–62 (2009).
44. A. Purushotham, T. T. Schug, Q. Xu, S. Surapureddi, X. Guo, X. Li, Hepatocyte-specific deletion of SIRT1 alters fatty acid metabolism and results in hepatic steatosis and inflammation. *Cell Metab.* **9**, 327–338 (2009).
45. Y. Zu, L. Liu, M. Y. Lee, C. Xu, Y. Liang, R. Y. Man, P. M. Vanhoutte, Y. Wang, SIRT1 promotes proliferation and prevents senescence through targeting LKB1 in primary porcine aortic endothelial cells. *Circ. Res.* **106**, 1384–1393 (2010).
46. A. S. Banks, N. Kon, C. Knight, M. Matsumoto, R. Gutierrez-Juárez, L. Rossetti, W. Gu, D. Accili, SIRT1 gain of function increases energy efficiency and prevents diabetes in mice. *Cell Metab.* **8**, 333–341 (2008).
47. J. B. Pillai, M. Chen, S. B. Rajamohan, S. Samant, V. B. Pillai, M. Gupta, M. P. Gupta, Activation of SIRT1, a class III histone deacetylase, contributes to fructose feeding-mediated induction of the α-myosin heavy chain expression. *Am. J. Physiol. Heart Circ. Physiol.* **294**, H1388–H1397 (2008).
48. T. H. Schreiber, D. Wolf, M. S. Tsai, J. Chirinos, V. V. Deyev, L. Gonzalez, T. R. Malek, R. B. Levy, E. R. Podack, Therapeutic Treg expansion in mice by TNFRSF25 prevents allergic lung inflammation. *J. Clin. Invest.* **120**, 3629–3640 (2010).
49. S. B. Rajamohan, V. B. Pillai, M. Gupta, N. R. Sundaresan, K. G. Birukov, S. Samant, M. O. Hottiger, M. P. Gupta, SIRT1 promotes cell survival under stress by deacetylation-dependent deactivation of poly(ADP-ribose) polymerase 1. *Mol. Cell. Biol.* **29**, 4116–4129 (2009).
50. N. R. Sundaresan, S. A. Samant, V. B. Pillai, S. B. Rajamohan, M. P. Gupta, SIRT3 is a stress-responsive deacetylase in cardiomyocytes that protects cells from stress-mediated cell death by deacetylation of Ku70. *Mol. Cell. Biol.* **28**, 6384–6401 (2008).

Downloaded from stke.sciencemag.org on September 26, 2011

51. M. P. Gupta, S. A. Samant, S. H. Smith, S. G. Shroff, HDAC4 and PCAF bind to cardiac sarcomeres and play a role in regulating myofilament contractile activity. *J. Biol. Chem.* **283**, 10135–10146 (2008).
52. V. B. Pillai, N. R. Sundaresan, G. Kim, M. Gupta, S. B. Rajamohan, J. B. Pillai, S. Samant, P. V. Ravindra, A. Isbatan, M. P. Gupta, Exogenous NAD blocks cardiac hypertrophic response via activation of the SIRT3-LKB1-AMP-activated kinase pathway. *J. Biol. Chem.* **285**, 3133–3144 (2010).
53. N. R. Sundaresan, M. Gupta, G. Kim, S. B. Rajamohan, A. Isbatan, M. P. Gupta, Sirt3 blocks the cardiac hypertrophic response by augmenting Foxo3a-dependent antioxidant defense mechanisms in mice. *J. Clin. Invest.* **119**, 2758–2771 (2009).
54. C. C. Milburn, M. Deak, S. M. Kelly, N. C. Price, D. R. Alessi, D. M. Van Aalten, Binding of phosphatidylinositol 3,4,5-trisphosphate to the pleckstrin homology domain of protein kinase B induces a conformational change. *Biochem. J.* **375**, 531–538 (2003).
55. **Acknowledgments:** We thank B.-C. He for his technical help in injecting cells in nude mice. We are also grateful to W. McBurney (University of Ottawa, Canada) for providing SIRT1-KO mice. Adenovirus vectors synthesizing shRNA against p300 and SIRT1 were provided by B. Thimmapaya (Northwestern University, Chicago, IL) and P. Puigserver (Harvard Medical School, Boston, MA), respectively. **Funding:** This study was supported by NIH grants RO1 HL-77788 and HL-83423 to M.P.G. N.R.S. was supported by a post-doctoral fellowship grant from the American Heart Association. **Author contributions:** Most of the experiments in this study were done by N.R.S. V.B.P. performed time course and cellular NAD/NADH ratio experiments. J.M.C. was involved in the planning of experiments related to cancer growth. D.W. did MS/MS analysis. S.S. was involved in creating Akt mutants and performed GST pull-down assays and localization experiments. P.V. and V.P. worked in the laboratory of J.M.C. and did experiments related to cancer cell growth and tumorigenesis. H.R. generated in silico models showing interaction of the PH domain of Akt and PDK1 with PIP_3. M.G. analyzed cardiac hypertrophy in mice. M.P.G. coordinated with different investigators and prepared the manuscript. **Competing interests:** The authors declare that they have no competing interests. **Data availability:** The MS data associated with this manuscript may be downloaded from the Proteome Commons Tranche repository at https://proteomecommons.org/tranche/ (Tranche hash: Ar624VS4wP/z/2MG7dPnqkgNsh0iEiT/3943A7xZPQcseJLI+2oV1fsnC7Ti0sqKiWK8l3b2E86cdwPfw6BgPQ2Tc+EAAAAAAAACFQ==).

Submitted 26 August 2010
Accepted 29 June 2011
Final Publication 19 July 2011
10.1126/scisignal.2001465
Citation: N. R. Sundaresan, V. B. Pillai, D. Wolfgeher, S. Samant, P. Vasudevan, V. Parekh, H. Raghuraman, J. M. Cunningham, M. Gupta, M. P. Gupta, The deacetylase SIRT1 promotes membrane localization and activation of Akt and PDK1 during tumorigenesis and cardiac hypertrophy. *Sci. Signal.* **4**, ra46 (2011).

Downloaded from stke.sciencemag.org on September 26, 2011