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Sirt2 Deacetylase Is a Novel AKT Binding Partner Critical for AKT Activation by Insulin*

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Background: AKT kinases mediate insulin signaling downstream of phosphatidylinositol-3 kinase (PI3K).
Results: AKT binds Sirt2 in insulin-responsive cells and Sirt2 inhibition blocks AKT activation, whereas Sirt2 overexpression sensitizes cells to insulin.
Conclusion: Sirt2 deacetylase is an essential factor in AKT activation.
Significance: Sirt2 modulators could be useful in treatment of diseases involving AKT, such as type 2 diabetes and cancer.

AKT/PKB kinases transmit insulin and growth factor signals downstream of phosphatidylinositol 3-kinase (PI3K). AKT activation involves phosphorylation at two residues, Thr^{308} and Ser^{473}, mediated by PDK1 and the mammalian target of rapamycin complex 2 (mTORC2), respectively. Impaired AKT activation is a key factor in metabolic disorders involving insulin resistance, whereas hyperactivation of AKT is linked to cancer pathogenesis. Here, we identify the cytoplasmic NAD$^+$-dependent deacetylase, Sirt2, as a novel AKT interactor, required for optimal AKT activation. Pharmacological inhibition or genetic down-regulation of Sirt2 diminished AKT activation in insulin and growth factor-responsive cells, whereas Sirt2 overexpression enhanced the activation of AKT and its downstream targets. AKT was prebound with Sirt2 in serum or glucose-deprived cells, and the complex dissociated following insulin treatment. The binding was mediated by the pleckstrin homology and the kinase domains of AKT and was dependent on AMP-activated kinase. This regulation involved a novel AMP-activated kinase-dependent Sirt2 phosphorylation at Thr^{101}. In cells with constitutive PI3K activation, we found that AKT also associated with a nuclear sirtuin, Sirt1; however, inhibition of PI3K resulted in dissociation from Sirt1 and increased association with Sirt2. Sirt1 and Sirt2 inhibitors additively inhibited the constitutive AKT activity in these cells. Our results suggest potential usefulness of Sirt1 and Sirt2 inhibitors in the treatment of cancer cells with up-regulated PI3K activity and of Sirt2 activators in the treatment of insulin-resistant metabolic disorders.

Regulation of cellular metabolism and glucose uptake by insulin is a highly complex and incompletely understood process, involving a large network of signaling proteins, metabolic enzymes, and transcriptional regulators (1, 2). Activation of the phosphatidylinositol 3-kinase (PI3K)-AKT/PKB pathway is considered the main signaling component downstream of the insulin receptor (3, 4). When activated, AKT regulates a multitude of targets by direct phosphorylation (5). AKT activation involves binding to inositol 1,4,5-trisphosphate at the plasma membrane, which induces a conformational change in the protein, facilitating its phosphorylation by its activating kinases PDK1 and mammalian target of rapamycin complex 2 (mTORC2)[2] (6). PDK1 phosphorylates AKT at Thr^{308} and mTORC2 at Ser^{473}. A fraction of activated AKT translocates to the nucleus and phosphorylates specific nuclear targets (7). The PI3K-AKT pathway is up-regulated in many cancers, where it promotes cell proliferation, survival, and drug resistance (8). Thus, the pathway is regarded as a promising therapeutic target for cancer treatment, and several inhibitors of the pathway are under evaluation in clinical trials in multiple cancer types (9, 10).

Sundaresan *et al.* (11) reported recently regulation of AKT and PDK1 by reversible acetylation. This work showed that p300 and pCAF acetylate AKT and PDK1, whereas Sirt1 deacetylates them. The acetylation regulated the ability of AKT to bind inositol 1,4,5-trisphosphate, offering a novel mode of AKT regulation through reversible acetylation (12).

Sirtuins are evolutionary conserved NAD$^+$-dependent protein deacetylases analogous to the yeast silent information regulator 2 (Sir2) (13–15). They are involved in the regulation of numerous physiological and cellular processes, including oxidative stress and DNA damage responses, metabolism, and aging. Mammalian sirtuins consist of seven family members, Sirt1 to -7, varying in their cellular localization and function. Sirt1, -6, and -7 are primarily nuclear, Sirt2 is cytoplasmic, and Sirt3, -4, and -5 are primarily mitochondrial (16). Sirt1 is the most studied isoform and has been shown to deacetylate a multitude of targets, including transcription factors, such as p53, NF-κB, and FoxO family members, and metabolic proteins,

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[2] The abbreviations used are: mTORC1 and -2, mammalian target of rapamycin complex 1 and 2, respectively; mTOR, mammalian target of rapamycin; AMPK, AMP-activated kinase.

such as PGC-1α, PPAR-γ, LXR, and SREBP1c (14, 17). Sirt1 knockout results in developmental defects and hyperacetylation of its targets, whereas Sirt1 overexpression counters some of the negative effects of high fat diet on glucose metabolism and liver damage (18, 19). Sirt2, the primary cytoplasmic sirtuin, has been attributed tumor suppressor functions and a role in maintaining genome integrity as well as a role in programmed necrosis (20–22). Among its deacetylation targets are FoxO transcription factors, tubulin, keratin 8, eIF5A, APC/C, and NF-κB-p65 (23–29). Controversy remains, however, regarding the role of Sirt2 in regulating tubulin acetylation in the brain (30).

There is substantial cross-talk between the insulin-PI3K-AKT-metabolic pathways and sirtuins (17, 31–33). For example, Sirt1 and Sirt2 can deacetylate and regulate the function of FoxO transcription factors, which are direct AKT targets (24, 26, 27, 34). However, the exact role of sirtuins in insulin responsiveness is still not clear and could be tissue-dependent, because hepatic Sirt1 deficiency, for example, was reported to lead to insulin resistance (35), whereas neuronal deficiency results in increased insulin sensitivity (36). Sirt1 was also reported to promote AKT phosphorylation in muscle and HEK-293 cells through interaction with the PI3K adapter subunit p85 (33).

Here, we demonstrate that the main sirtuin that binds and regulates AKT activation in insulin-responsive cells is Sirt2 rather than Sirt1. Whereas AKT associates with both Sirt1 and Sirt2 in cells with constitutive PI3K activation, it exclusively binds Sirt2 in cells with normal regulation of the PI3K-AKT pathway. The Sirt2-AKT binding is induced by glucose and nutrient depravation and PI3K inhibition, whereas insulin treatment induces the dissociation of the complex. AMPK activity, which is negatively regulated by insulin, is required for maintaining the AKT-Sirt2 complex, in part, through regulating Sirt2 phosphorylation at Thr^{101}. Sirt2 inhibition or its knockout diminishes AKT activation by insulin, whereas Sirt2 overexpression increases the activation of AKT and its downstream targets. Our study identifies Sirt2 as a new positive AKT regulator that potentiates insulin responsiveness in normal cells while demonstrating that both Sirt1 and Sirt2 play a role in maintaining AKT activation in cancer cells with constitutive PI3K activation. These findings suggest that Sirt2 activators could be useful in the treatment of obesity-associated metabolic syndrome and type 2 diabetes, whereas Sirt1/2 inhibitors may have therapeutic use in cancers with constitutive PI3K-AKT pathway activation.

EXPERIMENTAL PROCEDURES

cDNA Constructs, Antibodies, and Inhibitors—pExchange 5A Sirt1-FLAG and Sirt2-FLAG were constructed by PCR amplification of Sirt1 and Sirt2 from pCDNA-DEST47-GFP-Sirt1 and pCDNA-DEST47-GFP-Sirt2 (a gift from Izumi Horikawa) (16) and subcloning into the pExchange 5A-FLAG vector (Stratagene). The QuikChange site-directed mutagenesis kit (Stratagene) was used to generate the T101A and T101D Sirt2 mutants. pEBG-GST-AKT and its inactive or constitutive mutants were described previously (37). Expression vectors for the GST-AKT fragments were a gift from Keqiang Ye (38). pEBG-GST-AMPK-1–312, encoding for a constitutively active AMPK, was a gift from John Kyriakis. Pan-AKT, $Ser(P)^{473}$-AKT, $Thr(P)^{308}$-AKT, $Ser(P)^{389}$-p70-S6K, $Thr(P)^{202}/Tyr(P)^{204}$-ERK, ERK, $Ser(P)^{9}$-GSK3β, GSK3β, ac-K40-α-tubulin, α-tubulin, GST, β-actin, HSP90, and GAPDH were from Cell Signaling Technologies. Sirt1, Sirt2, and FLAG were from Sigma. LY294002, compound C, AGK-2, AK-1, and AK-7 were from Selleck Chemicals.

Cell Culture and Transfection—HEK-293T, NIH-3T3, COS-7, HeLa, 3T3-L1, MDA-MB-468, wild type, and Sirt2-null mouse embryo fibroblasts, a kind gift from Chu-Xia Deng (20), were maintained in high glucose DMEM (Invitrogen), supplemented with 10% fetal bovine serum in 5% CO_2. CHO-K1 cells were maintained in Ham's F-12 medium. Transient transfections were performed using FuGENE HD (Roche Applied Science) according to the manufacturer's instructions.

Cell Extraction, Fractionation, and Western Blot Analysis—Cellular protein was extracted in lysis buffer containing 50 mM Tris-Cl, pH 7.5, 100 mM NaCl, 1% Triton, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 50 mM β-glycerol phosphate, 2 mM Na_2VO_4, and protease inhibitors and cleared by centrifugation. The NE-PER fractionation kit (Pierce) was used for nuclear/cytoplasmic fractionation according to the manufacturer's instructions. Protein extracts were separated using SDS-PAGE, transferred to 0.45 μM Immobilon-P PVDF membrane (Millipore), and immunoblotted using the indicated antibodies followed by ECL. The blots were analyzed using ChemiDoc digital imaging system (Bio-Rad).

Immunoprecipitation and Protein Binding Assays—Cells expressing the indicated vectors were lysed, and proteins were extracted as detailed above. Equal amounts of protein (1–2 mg/point) were incubated with protein A/G-agarose beads (Santa Cruz Biotechnology, Inc.) coupled with the appropriate antibody or with GSH-Sepharose beads (GE Healthcare) for GST purification for 90 min followed by two washes with lysis buffer, one wash with lysis buffer containing 0.5 M LiCl, and two washes with buffer containing 40 mM Tris-Cl, pH 7.5, 0.1 mM EDTA, and 5 mM $MgCl_2$. For the last wash, the beads were transferred to a new tube, and the proteins were eluted using SDS-PAGE sample buffer at 95 °C for 5 min. Total cell extracts from each sample were saved to examine protein expression and pathway activation/inhibition.

^{32}P Metabolic Labeling and Two-dimensional Phosphopeptide Mapping—Transfected cells were serum-deprived for 16 h, washed once with medium lacking phosphate, and incubated for 30 min in phosphate-free medium supplemented with the indicated inhibitors. Cells were radiolabeled in the presence of 0.5 mCi/ml ^{32}P for 2 h, and protein extracts were used for immunoprecipitation. The two-dimensional phosphopeptide mapping was performed as described previously (39). Briefly, the immunopurified ^{32}P-labeled Sirt2 was resolved using 10% SDS-PAGE, transferred to a PVDF membrane, and excised, and ^{32}P incorporation was determined by Cherenkov counting. Following incubation with 0.5% polyvinyl phosphate in 100 mM acetic acid for 30 min at 37 °C and five washes with DDW and two with 50 mM ammonium bicarbonate buffer, Sirt2 protein samples were digested with 10 μg of sequencing grade modified trypsin (Promega) for 2 h at 37 °C and with an additional 10 μg of trypsin for 12 h. Following elution, the digested peptides

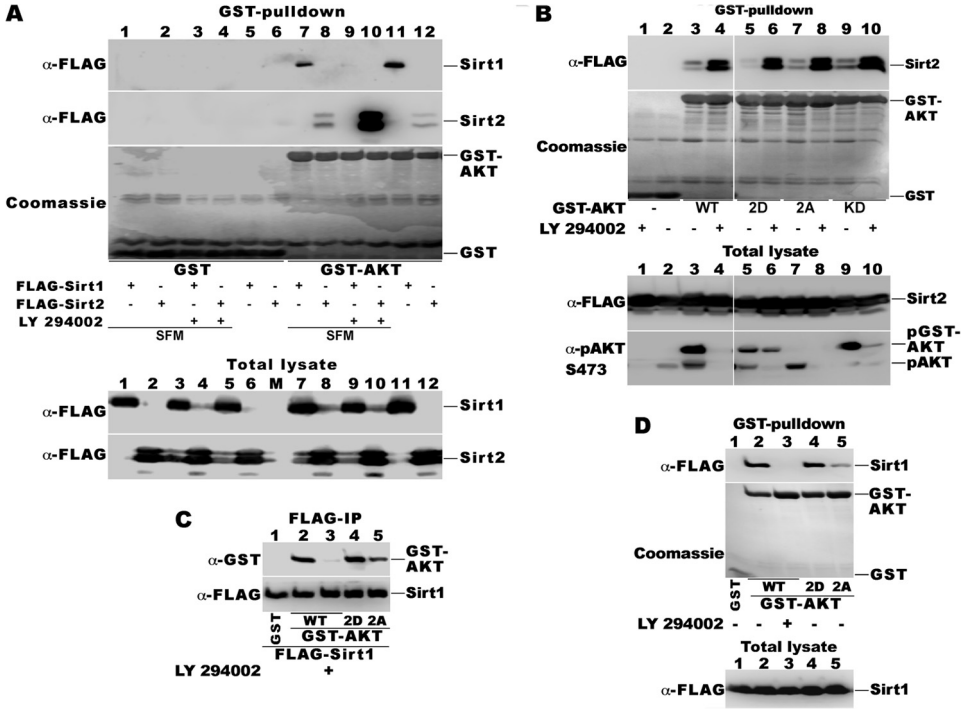

FIGURE 1. **Sirt1 and Sirt2 alternate in AKT binding in response to PI3K inhibition in HEK-293T cells.** *A*, HEK-293T cells were transfected with GST control or GST-AKT together with FLAG-Sirt1 or Sirt2 as indicated. Exponentially growing or serum-deprived cells (*SFM*) were treated with vehicle or with a 10 μM concentration of the PI3K inhibitor, LY 294002, for 14 h. FLAG-sirtuin co-purification in GST pull-downs and its expression in total cell lysates were analyzed by FLAG immunoblotting. GST and GST-AKT recovery was analyzed by Coomassie staining. *B*, HEK-293T cells were transfected with FLAG-Sirt2 together with GST control or GST-AKT (wild type (*WT*), T308D/S473D (*2D*), T308A/S473A (*2A*), or K178M (*KD*)), and AKT-Sirt2 association was analyzed as in *A*. Total lysates were immunoblotted for phospho-AKT (Ser^{473}) to determine AKT activation. *C* and *D*, HEK-293T cells were transfected with FLAG-Sirt1 together with GST control or the indicated GST-AKT forms, and AKT-Sirt1 association was examined as in *A* (*D*) or after FLAG-immunoprecipitation (*IP*) and GST immunoblotting (*C*).

were washed twice with 50 mM ammonium bicarbonate buffer and once with pH 1.9 TLC electrophoresis buffer (2.2% formic acid and 7.8% acetic acid in water). Samples were spotted on cellulose TLC plates (Merck) and separated using the Hunter thin layer chromatography system (CBS Scientific) in pH 1.9 buffer for 25 min at 1000 V. The plates were dried overnight and subjected to second dimension chromatographic separation in a phosphochromatography buffer (37.5% *n*-butanol, 25% pyridine, and 7.5% acetic acid). The plates were dried, and the phosphopeptide spots were visualized by autoradiography and phosphorimaging.

Immunofluorescence Staining and Confocal Microscopy—HeLa cells transiently expressing AKT and sirtuins were fixed using 4% paraformaldehyde for 20 min and stained for AKT (rabbit) or FLAG (mouse) for Sirt1 and Sirt2. Propidium iodide was used for nuclear staining. Localization of AKT and sirtuins was analyzed using confocal microscopy (Nikon).

RESULTS

PI3K Activity Coordinates Alternate AKT Binding with Sirt1 and Sirt2 in HEK-293T Cells—In our effort to identify sirtuin regulators, we detected binding of Sirt1 and Sirt2 with AKT in HEK-293T cells (Fig. 1*A*). However, their binding to AKT was oppositely regulated by PI3K activity; Sirt1 associated with AKT in exponentially growing cells, when the PI3K-AKT pathway is constitutively active in HEK293T cells due to the expression of the large T antigen, but dissociated following PI3K inhibition (Fig. 1*A*, compare *lanes 9* and *11*). In contrast, PI3K inhibition enhanced Sirt2 binding to AKT (Fig. 1*A*, compare *lanes 10* and *12*). To examine whether this switch was mediated by changes in the AKT activation state, we tested the binding of Sirt2 (Fig. 1*B*) and Sirt1 (Fig. 1, *C* and *D*) to constitutively active (T308D/S473D) and inactive (T308A/S473A and K178M) AKT forms. Sirt2 binding to AKT remained responsive to PI3K inhibition regardless of the AKT activation state (Fig. 1*B*), indicating that the PI3K activity affects the complex in a manner independent of AKT activation. In contrast, Sirt1 showed low binding with the phosphorylation-deficient AKT form, T308A/S473A (Fig. 1, *C* and *D*, compare *lanes 2* and *5*), suggesting that Sirt1 preferentially binds to active AKT.

AKT Binds Sirt2 but Not Sirt1 in Insulin-responsive Cells with Normal AKT Regulation—Because HEK-293T cells exhibit abnormal, constitutive PI3K-AKT activation, we reexamined the regulation of AKT binding to Sirt1 and Sirt2 in cells displaying normal, insulin-regulated AKT activation. Sirt2 abundantly bound AKT in serum-deprived CHO-K1 cells that display low AKT activity, and PI3K inhibition did not affect the association level (Fig. 2*A*), indicating that the effect of PI3K inhibition observed in HEK-293T cells was related to their high basal PI3K activity. Insulin treatment induced dissociation of the Sirt2-AKT complex (Fig. 2*A*, compare *lane 8* with *lanes 12* and *14*), which is consistent with the reduced binding observed in HEK-293T cells, and confirms that AKT preferentially binds Sirt2 when the insulin-PI3K pathway activity is low. To our surprise, however, we were unable to detect any binding of AKT with Sirt1 in CHO-K1 cells, even after prolonged stimulation with insulin (Fig. 2*A*, *lanes 11* and *13*). Excluding the possibility that

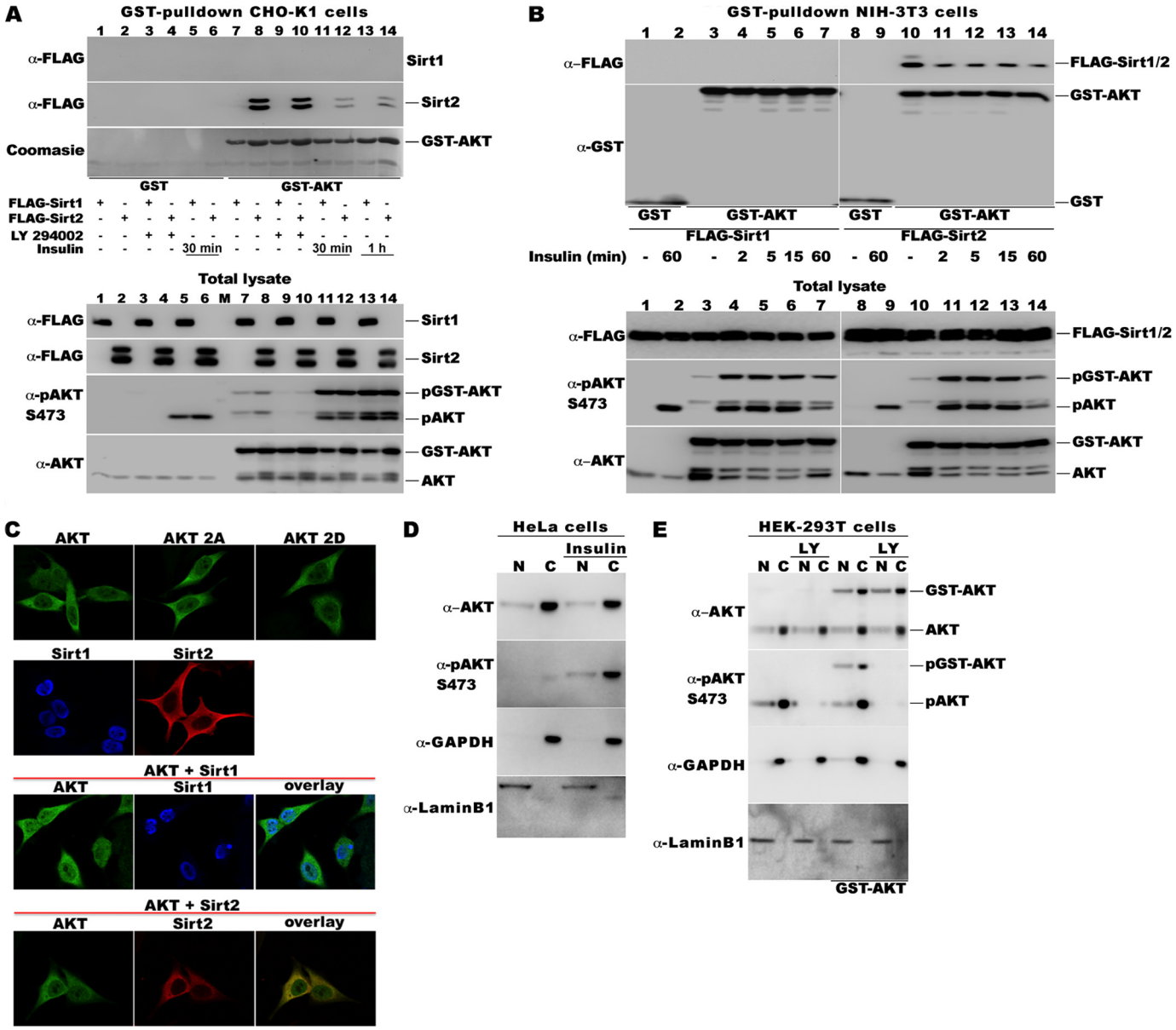

FIGURE 2. **Sirt2, but not Sirt1, is found in a complex with AKT in insulin-responsive cells.** *A* and *B*, CHO-K1 (*A*) or NIH 3T3 (*B*) cells were transfected with GST control or GST-AKT together with FLAG-Sirt1 or Sirt2 as indicated. Serum-deprived cells were treated with vehicle or with 10 μM LY 294002 for 14 h (*A*) or with 1 μM insulin for the indicated times (*A* and *B*). AKT-sirtuin binding was analyzed as in Fig. 1*A*. Total lysates were immunoblotted for phospho-AKT and total AKT for determining AKT activation. *C*, HeLa cells expressing the indicated AKT forms or coexpressing AKT with FLAG-Sirt1 or FLAG-Sirt2 were stained for AKT or FLAG and analyzed using confocal microscopy. Shown are representative pictures at ×600 magnification. *D* and *E*, HeLa cells (*D*) or HEK-293T cells expressing GST control or GST-AKT (*E*) were treated for 15 min with 1 μM insulin (*D*) or for 2 h with 10 μM LY 294002 (*E*), and the cells were fractionated. The presence of total and phospho-AKT in nuclear and cytoplasmic fractions was analyzed by immunoblotting. GAPDH and lamin B1 immunoblots were used for confirming fractionation purity.

this was a cell type-specific phenomenon, similar results were obtained in other insulin-responsive cell lines with low basal PI3K-AKT activity, including NIH 3T3 (Fig. 2*B*) and COS-7 cells (data not shown), suggesting that Sirt2 and not Sirt1 is the main AKT binding partner in cells with physiological regulation of the PI3K-AKT signaling pathway. These results suggest that AKT-Sirt1 binding may happen mainly under chronic constitutive activation of the PI3K-AKT pathway, such as the one found in cancer cells with PTEN inactivation or cancers with activated tyrosine kinase receptors.

Sirt1 was reported to be primarily nuclear, and Sirt2 was reported to be cytoplasmic (16), whereas AKT shuttles to the nucleus following activation (7). To examine whether the effects of insulin and PI3K on AKT binding with Sirt1 and Sirt2 involved changes in their subcellular localization, we analyzed the localization of AKT and the sirtuins in HEK-293T and HeLa cells (Fig. 2). Confirming the previous reports, we found that in HeLa cells, Sirt1 was strictly nuclear, whereas Sirt2 was cytoplasmic (Fig. 2*C*). AKT localized primarily to the cytoplasm but was also detectable in the nucleus (Fig. 2, *C* and *D*). The activation level of AKT did not seem to affect its localization because active and inactive AKT mutants showed similar localization patterns (Fig. 2*C*), and insulin treatment did not affect the subcellular distribution (Fig. 2*D*). We obtained similar localization

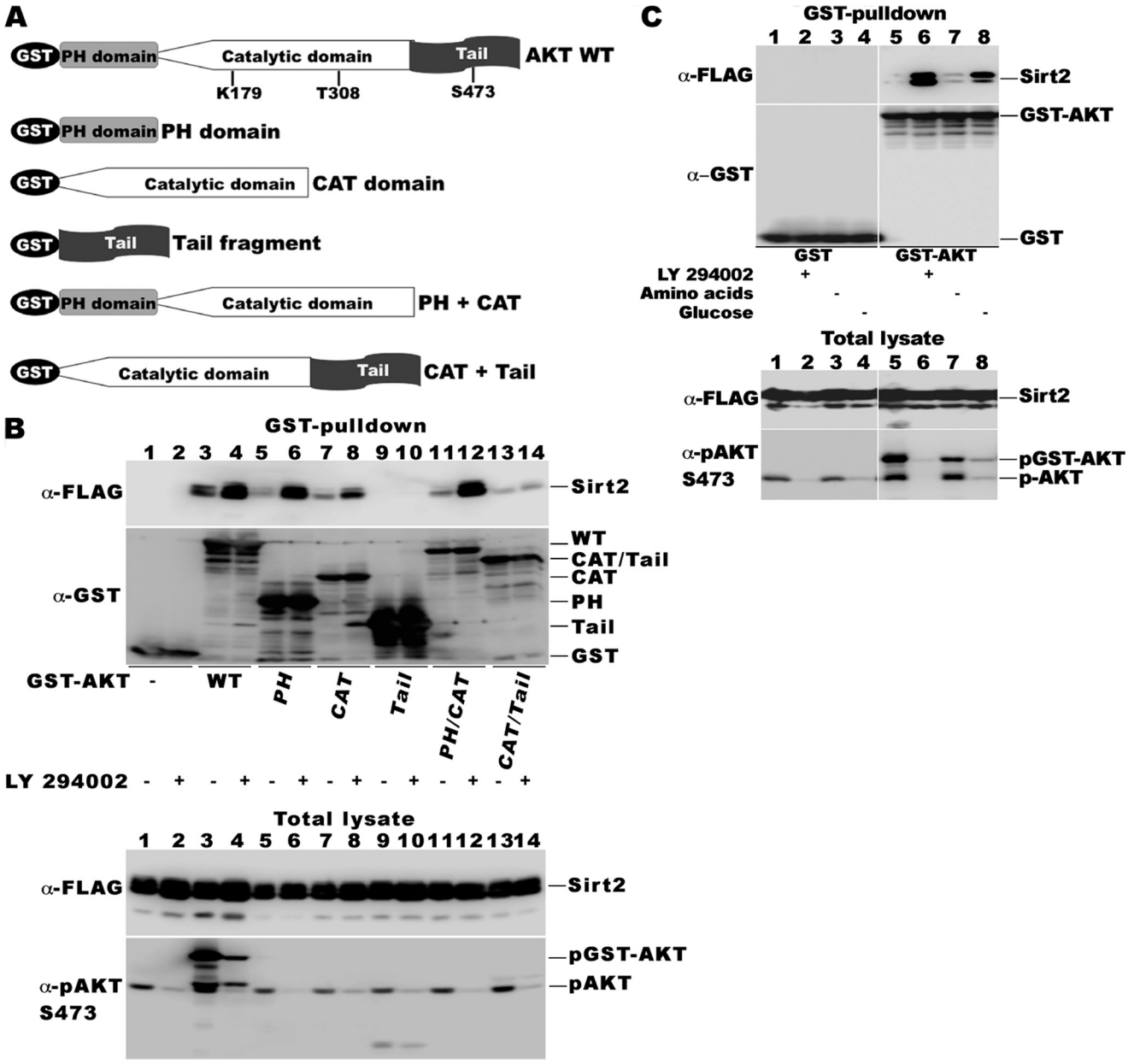

FIGURE 3. **The pleckstrin homology (*PH*) and catalytic domains of AKT mediate the binding to Sirt2.** *A*, schematic representation of the GST-AKT fragments used in this study. Noted are the activating phosphorylation sites and the key lysine in the ATP-binding pocket. *B*, HEK-293T cells were transfected with GST control or the indicated GST-AKT fragments together with FLAG-Sirt2. Cells were treated with vehicle or with 10 μM LY 294002 for 14 h as indicated. AKT-Sirt2 binding was analyzed as in Fig. 1*A*. Total lysates were immunoblotted for FLAG and phospho-AKT. *C*, HEK-293T cells co-expressing GST control or GST-AKT with FLAG-Sirt2 were treated with vehicle or with 10 μM LY 294002 or were deprived of glucose or amino acids for 14 h as indicated. AKT-Sirt2 binding was analyzed as above.

results in HEK-293T cells, and PI3K inhibition did not affect the subcellular distribution of AKT (Fig. 2*E*). These results suggest that the observed effects of insulin and PI3K inhibition on AKT binding with Sirt1 and Sirt2 are not mediated by changes in their subcellular localization.

The Pleckstrin Homology and Catalytic Domains of AKT Mediate the Binding to Sirt2—To better characterize the AKT-Sirt2 association, we examined the binding of Sirt2 to functional AKT domains (Fig. 3, *A* and *B*). These experiments identified strong Sirt2 binding with the pleckstrin homology and the catalytic domains of AKT, which remained responsive to PI3K inhibition (Fig. 3*B*, compare *lane 5* with *lane 6* and *lane 7* with *lane 8*). There was no binding with the tail region, and its inclusion with the catalytic domain diminished the binding (Fig. 3*B*, compare *lanes 7* and *8* with *lanes 13* and *14*).

To identify a physiological setting corresponding to the observed PI3K inhibition-induced association of Sirt2 with AKT in HEK-293T cells, we examined the effects of nutrient deprivation on Sirt2-AKT association (Fig. 3*C*). These experiments showed that glucose deprivation and, to some extent, amino acid deprivation induced Sirt2-AKT association. The ability of insulin to dissociate the AKT-Sirt2 complex and of glucose deprivation and PI3K inhibition to induce complex formation independent of the AKT activation state suggested to us

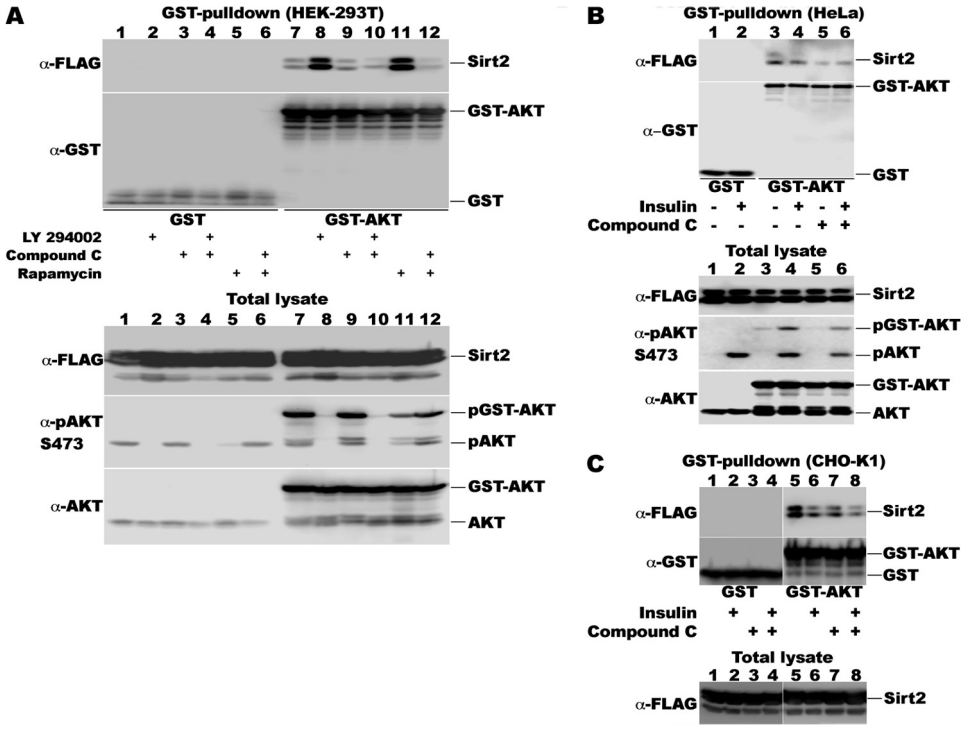

FIGURE 4. **AMPK regulates Sirt2-AKT association.** *A*, HEK-293T cells co-expressing Sirt2 with GST control or GST-AKT were treated with 10 μM LY 294002, a 1 μM concentration of the AMPK inhibitor compound C, or 200 nM rapamycin for 14 h or their combinations, as indicated, and AKT-Sirt2 binding was analyzed as in Fig. 1*A*. *B* and *C*, HeLa (*B*) or CHO-K1 (*C*) cells transfected as in *A* were pretreated with 1 μM compound C for 1 h, followed by 1 μM insulin for 30 min as indicated, and AKT-Sirt2 binding was analyzed.

that the regulation of the complex might involve direct effects of the PI3K pathway on Sirt2.

Sirt2 Binding to AKT Requires AMPK Activity and Involves Sirt2 Phosphorylation at Thr^{101}—Given that AMP-activated protein kinase (AMPK) and mTOR sense glucose and nutrient availability and mediate insulin signals downstream of PI3K (4), we tested the effects of the AMPK inhibitor dorsomorphin/compound C and of the mTORC1 inhibitor rapamycin on AKT-Sirt2 binding (Fig. 4). In HEK-293T cells, compound C did not have an effect on the basal Sirt2-AKT binding; however, it blocked the ability of PI3K inhibition to induce the binding (Fig. 4*A*, compare *lanes 8* and *10*). Because the PI3K-AKT pathway negatively regulates AMPK, the result suggests that AMPK activation in response to PI3K inhibition is involved in the enhanced Sirt2-AKT binding. This result also confirmed our initial observation that the AKT activation state *per se* was not involved in conferring the binding to Sirt2, because the combined PI3K/AMPK inhibitor-treated cells had negligible AKT phosphorylation (Fig. 4*A*, *lane 10*). Rapamycin treatment, on the other hand, induced AKT binding with Sirt2 as efficiently as PI3K inhibition (Fig. 4*A*, compare *lanes 7* and *11*), indicating involvement of mTORC1 in the regulation of the binding. This enhancement was also blocked by compound C, suggesting that AMPK is the main regulator of AKT-Sirt2 binding downstream of the PI3K-AKT-mTORC1 pathway. In agreement with the results obtained in HEK-293T cells, AMPK inhibition in HeLa (Fig. 4*B*) and CHO-K1 cells (Fig. 4*C*) was able to repress the basal Sirt2-AKT binding similarly to insulin treatment. Because insulin treatment leads to AMPK inhibition (40), our results suggest that insulin could be inducing the AKT-Sirt2 dissociation through inhibition of AMPK.

To examine whether AMPK acts directly on Sirt2, we used the MIT Scansite software that predicts protein phosphorylation sites and protein interaction motifs (41) to find potential AMPK phosphorylation sites in Sirt2, identifying one putative phosphorylation site at Thr^{101} (Fig. 5*A*). We performed *in vivo* ^{32}P-metabolic labeling experiments in the presence or absence of compound C and analyzed the two-dimensional phosphopeptide maps of wild type and T101A Sirt2 mutant to examine whether AMPK is involved in phosphorylation of Sirt2 at Thr^{101} (Fig. 5, *B* and *C*). AMPK inhibition resulted in reduced Sirt2 phosphorylation, both of wild type and of the Thr^{101} mutant (Fig. 5*B*, compare *lanes 1* and *3* with *lanes 2* and *4*). Analysis of the two-dimensional phosphopeptide maps of these samples showed that AMPK inhibition results in decreased phosphorylation at several sites (note that we loaded equal counts/min of the samples to allow stoichiometric comparison of phosphorylated peptides), with two spots showing marked reduction (Fig. 5*C*, compare *panels 5* and *6*; the spots are indicated with *arrows*). One of these spots was absent in the two-dimensional phosphopeptide map of the T101A mutant obtained from exponentially growing cells (Fig. 5*C*, *panel 7*), indicating its correspondence to the Thr^{101} site. The reduced phosphorylation observed at several other spots in the maps of the T101A mutant could be due to the presence of other phosphorylatable residues in the tryptic peptide containing the Thr^{101} site and may represent doubly and triply phosphorylated forms of the same peptide. Overall, these experiments identified Sirt2 Thr^{101} as a novel AMPK-regulated phosphorylation site and indicated that AMPK activity affected the phosphorylation of additional Sirt2 sites, directly or indirectly.

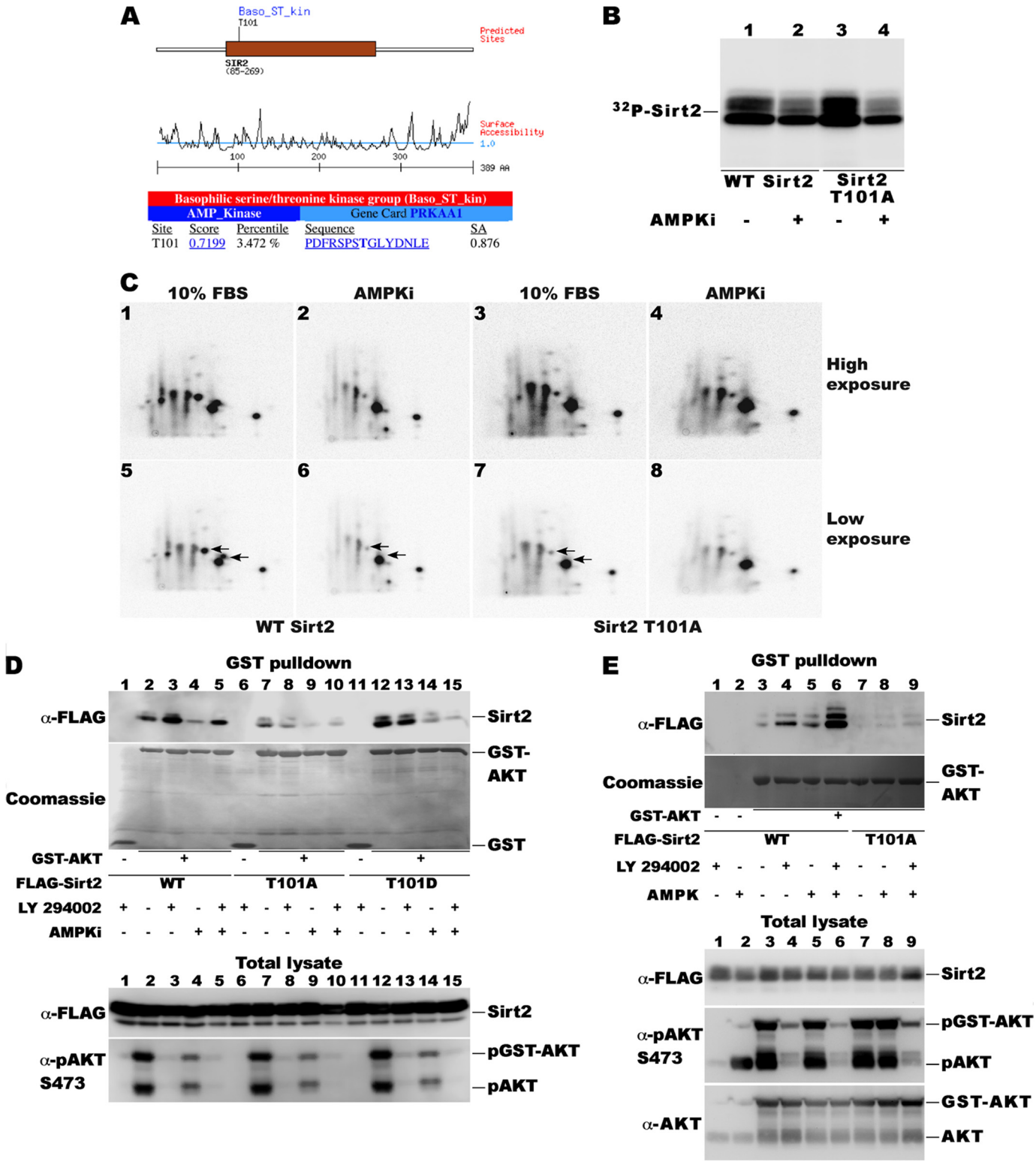

FIGURE 5. **Sirt2 Thr^{101} is an AMPK phosphorylation site involved in AKT binding.** *A*, schematic showing the identification of Sirt2 Thr^{101} as a potential AMPK phosphorylation site using the ScanSite program. Note the presence of other potential phosphorylation sites in the tryptic peptide containing Thr^{101}. *B* and *C*, HEK-293T cells expressing FLAG-Sirt2 were grown in 10% serum (FBS) and were treated with vehicle or 1 μM compound C (*AMPKi*) for 1 h before and during metabolic labeling with ^{32}P. Sirt2 was immunoprecipitated using FLAG antibody, and ^{32}P incorporation in Sirt2 was visualized (*B*). Two-dimensional phosphopeptide maps of Sirt2 were performed as described under "Experimental Procedures" (*C*). Note that equal counts/min of the samples were loaded to allow stoichiometric comparison of the phosphopeptides. Presented are high and low exposures of the maps. *Arrows* mark the two spots that are markedly decreased in the T101A mutant (see "Results" for more details). *D* and *E*, HEK-293T cells were transfected with GST control or GST-AKT together with wild type FLAG-Sirt2 or T101A or T101D mutants (*D*) or together with a constitutively active AMPK (*E*), and the cells were treated for 14 h with 10 μM LY 294002 or 1 μM compound C (*AMPKi*) or their combination, as indicated. AKT-Sirt2 binding was analyzed as in Fig. 1*A*.

To determine the functional role of Thr^{101} phosphorylation in Sirt2-AKT association, we compared the binding of AKT with wild type Sirt2, Sirt2 containing the phosphorylation-abrogating T101A mutation, and Sirt2 T101D, which introduces a charged residue at the site, mimicking phosphorylation (Fig. 5*D*). This analysis demonstrated that the PI3K inhibition-induced association of Sirt2 with AKT was largely dependent on Sirt2 phosphorylation state at the Thr^{101} site, because the T101A mutation resulted in low basal binding that lost responsiveness to PI3K inhibition (Fig. 5*D*, compare *lanes 2* and *3* with *lanes 7* and *8*), whereas the phosphomimetic T101D mutation resulted in increased basal binding that also lost responsiveness to PI3K inhibition (Fig. 5*D*, compare *lanes 2* and *3* with *lanes 12* and *13*). The ability of AMPK inhibition to suppress the binding of the Thr^{101} mutants suggests that AMPK inhibition could affect the binding by reducing phosphorylation of other Sirt2 sites, as observed in the ^{32}P-labeling experiments. Confirming the role of AMPK in regulating the binding, expression of a constitutively active AMPK-α1 form (1–312 catalytic region) resulted in a marked increase in AKT-Sirt2 association (Fig. 5*E*). This increase was not seen with the Thr^{101} Sirt2 mutant, suggesting that the effect of AMPK involved the Thr^{101} site.

Sirt2 Is a Critical Regulator of Insulin-induced AKT Activation—Next, we wanted to establish the physiological role of the Sirt2-AKT interaction. Initially, we examined whether Sirt2 or Sirt1 was a potential phosphorylation target of AKT. Analysis of their protein sequences for the presence of consensus AKT phosphorylation sites using the ScanSite program did not reveal candidate AKT phosphorylation sites in either protein. Nevertheless, we examined their phosphorylation by AKT in an *in vitro* kinase assay, using FoxO3 as a positive control and did not observe phosphorylation of either protein.[3]

To examine the potential involvement of Sirt2 in AKT activation, we tested the effects of specific Sirt2 inhibitors on AKT activation in insulin-responsive cells. We found that the specific Sirt2 inhibitor, AGK-2 (42), robustly attenuated the ability of insulin to activate AKT in 3T3-L1 preadipocytes (Fig. 6, *A* and *B*) and HeLa cells (Fig. 6*C*). This inhibition was especially apparent at low insulin concentrations (Fig. 6*B*; up to 100 nM) and was less significant at very high concentrations. This result indicates that at the supraphysiological concentrations that are often used in tissue culture systems, reaching sometimes up to 10 μM, the role of Sirt2 in AKT activation could be overlooked. Of note, the physiological concentration of insulin in plasma is below 1 nM. The effects of AGK-2 were not restricted to insulin, because it also diminished EGF-induced AKT activation in HeLa cells (Fig. 6*D*). Importantly, AGK-2 did not affect the ability of EGF to activate ERK in these experiments, pointing to the specificity toward the AKT pathway.

The specificity of Sirt2 inhibition was also addressed by using two other structurally unrelated Sirt2 inhibitors, AK1 and AK7, which showed results similar to those with AGK-2 (Fig. 7*A*). These experiments also showed that Sirt2 inhibition affected AKT phosphorylation at both of its activation sites, Thr^{308} and Ser^{471}. Interestingly, the Sirt1-specific inhibitor, CHIC35, did not have much effect on the insulin-induced AKT phosphorylation in HeLa cells (Fig. 7*B*, compare *lanes 6–8* with *lanes 10–12*). Supporting the results obtained with the Sirt2 inhibitors, mouse embryo fibroblast cells generated from a Sirt2 knock-out mouse (20) had reduced AKT phosphorylation in response to insulin compared with wild type mouse embryo fibroblasts (Fig. 7*C*).

To further confirm the role of Sirt2 in insulin-induced AKT activation, we generated stable HeLa cell lines overexpressing Sirt2 and compared their insulin responsiveness with that of the parental HeLa cells (Fig. 7, *D* and *E*). These experiments showed that Sirt2 overexpression results in increased AKT phosphorylation following insulin treatment, supporting the results obtained using Sirt2 inhibitors and Sirt2 knockout. Importantly, Sirt2-overexpressing cells also showed increased phosphorylation of AKT targets, such as GSK3β (Fig. 7*D*) and p70-S6-kinase (Fig. 7*E*), indicating that the increase in AKT phosphorylation reflects increased kinase activity.

Sirt1 and Sirt2 Inhibitors Additively Block AKT Activity in Cancer Cells with Constitutive PI3K Activation—To test whether the Sirt1-AKT and Sirt2-AKT interactions had significance in maintaining the constitutive high basal AKT activity in cells with impaired PI3K activation, we examined the effects of Sirt1 and Sirt2 inhibitors and their combination on AKT activity in HEK-293T (Fig. 8*A*) and in MDA-MB-468 breast cancer cells (Fig. 8*B*) that carry PTEN deletion and an activating mutation in PI3K. Inhibition of Sirt1 and, to a similar extent, of Sirt2 resulted in a significant decrease in AKT phosphorylation in both cell lines (Fig. 8, *A* and *B*, *lanes 3* and *4*), and combining both inhibitors further decreased the phosphorylation. The dietary compound, nicotinamide, that inhibits both Sirt1 and Sirt2, was also able to abolish AKT phosphorylation (Fig. 8, *A* (*lanes 6–8*) and *B* (*lane 6*)), supporting the data obtained with the specific inhibitors.

To get a better understanding of the role of Sirt2 in maintaining the high AKT activity in cells with impaired PI3K regulation, we examined the effect of Sirt2 inhibition on AKT activation after washout of the PI3K inhibitor (Fig. 8*C*, compare *lanes 1–7* with *lanes 8–14*). These experiments demonstrated that Sirt2 was required for optimal AKT activation by constitutive PI3K signaling, suggesting that Sirt2 inhibitors could synergize with PI3K inhibitors in treating cancer cells with impaired PI3K signaling.

DISCUSSION

Insulin-induced AKT activation requires AKT binding to inositol 1,4,5-trisphosphate at the membrane, which results in a conformational change in AKT that allows its phosphorylation by PDK1 and mTORC1 at Thr^{308} and Ser^{471}, respectively (3, 4, 6). The results presented in this paper add a new layer of regulation to this process that depends on Sirt2 activity. Our results also indicate that the constitutive pathologic activation of AKT found in cancer cells with up-regulated PI3K activation requires the activities of both Sirt1 and Sirt2. As illustrated in Fig. 8*D*, we propose that in cells displaying normal insulin-regulated AKT activation, AKT is found associated with Sirt2 but not with Sirt1. Under these conditions, Sirt2 activity is essential

[3] G. Ramakrishnan, G. Davaakhuu, L. Kaplun, W.-C. Chung, A. Rana, A. Atfi, L. Miele, and G. Tzivion, unpublished observation.

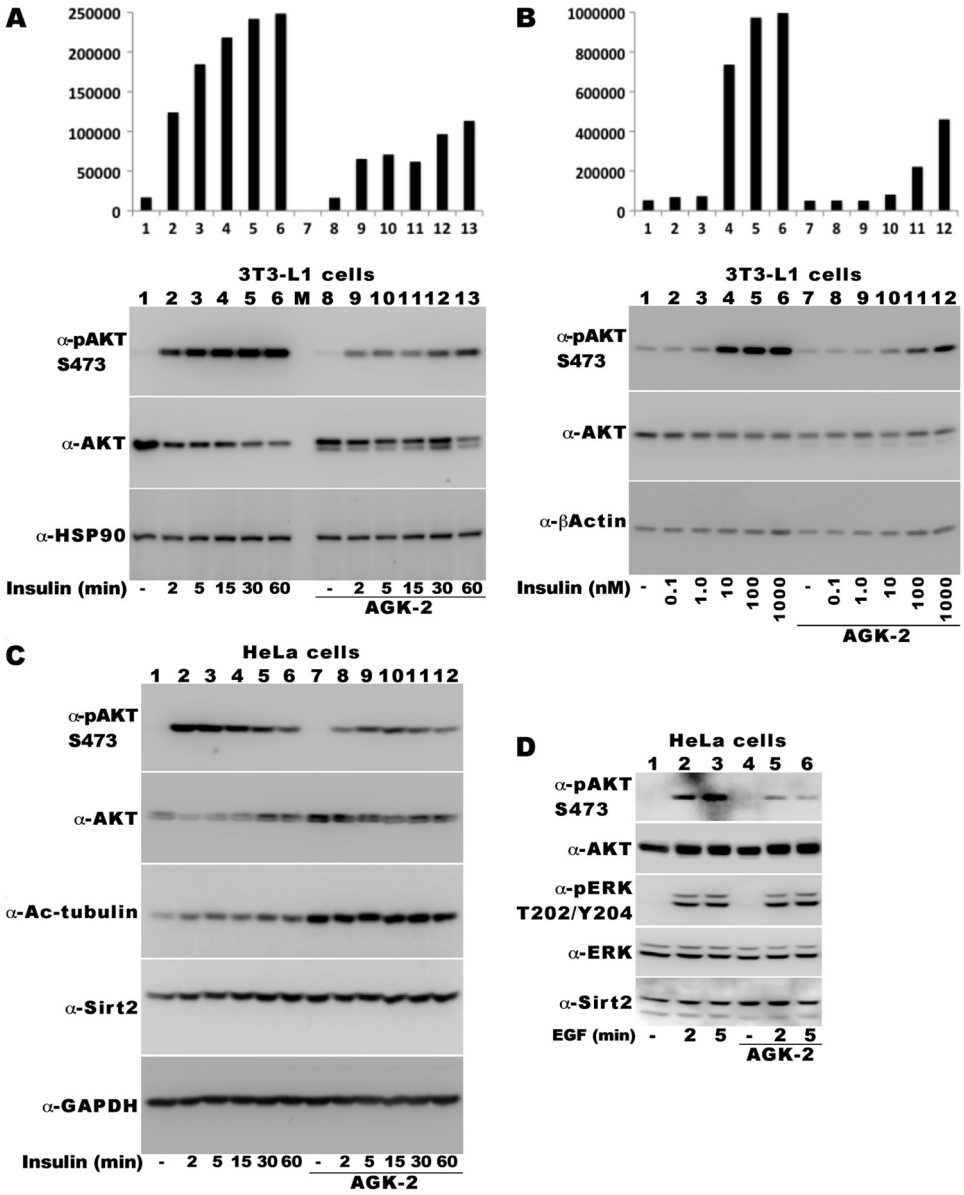

FIGURE 6. **Sirt2 inhibition attenuates AKT activation by insulin and EGF.** *A* and *B*, 3T3-L1 cells were treated with vehicle or a 2.5 μM concentration of the Sirt2 inhibitor AGK-2 for 1 h before the addition of 100 nM insulin for the indicated times (*A*) or for 15 min with the indicated insulin concentrations (*B*). AKT phosphorylation at Ser^{473} and total AKT were detected by immunoblotting. HSP90 (*A*) and β-actin (*B*) served as protein loading controls. pAKT band intensity quantification using Bio-Rad Quantity One software is provided as a *bar graph*. *C* and *D*, the effect of AGK-2 on AKT activation in HeLa cells in response to insulin (*C*) and EGF (50 ng/ml for 15 min; *D*) was examined as in *A*. Acetylation of tubulin on Lys^{40} served for demonstrating Sirt2 inhibition. ERK phosphorylation shows the specificity of AGK-2 for the AKT pathway.

for optimal AKT activation by insulin and growth factors. The association of AKT and Sirt2 is dependent on AMPK activity and involves phosphorylation of Sirt2 at Thr^{101}. Insulin-induced PI3K-AKT activation has been reported to inhibit AMPK (40). This effect could explain the dissociation of the AKT-Sirt2 complex following insulin treatment in insulin-responsive cells as well as the low basal binding we observed in HEK-293T cells with constitutive PI3K-AKT activation. The ability of rapamycin to increase the complex formation suggests that mTORC1 is involved in this regulation. Our results indicate that the AKT activation state does not affect the binding with Sirt2. However, whether the dissociation of the complex plays a role in AKT activation or function remains to be determined.

In cells with up-regulated PI3K activation, AKT is found associated also with Sirt1, and this binding is dependent on the high PI3K activity. When PI3K activity is inhibited, AKT dissociates from Sirt1 and increases its association with Sirt2. The binding to Sirt2 is sensitive to AMPK inhibition, mirroring the situation in insulin-responsive cells. In the high PI3K cancer cells, Sirt1 and Sirt2 inhibitors additively reduce AKT activity, reaching almost complete inhibition when combined or when the cells are treated with nicotinamide that inhibits both sirtuins.

Sundaresan *et al.* (11) reported recently the identification of AKT acetylation in the pleckstrin homology domain at residues Lys^{14} and Lys^{20}. Acetylation at Lys^{20} negatively regulated AKT activation by restricting the binding of AKT to inositol 1,4,5-

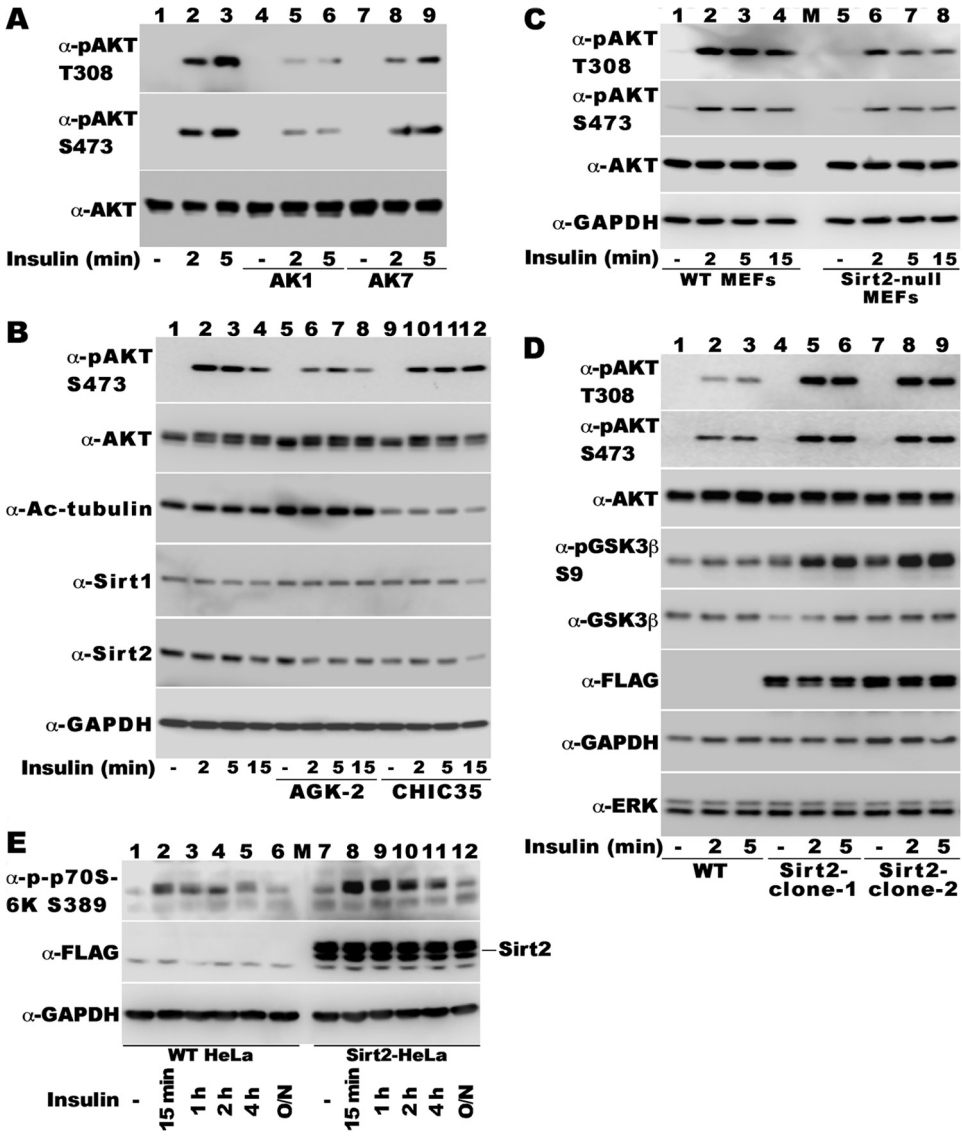

FIGURE 7. **Sirt2 overexpression sensitizes cells to insulin.** *A* and *B*, HeLa cells were pretreated for 2 h with a 10 μM concentration of the Sirt2 inhibitors AK1 and AK7 (*A*) or 2.5 μM AGK-2 or a 0.5 μM concentration of the Sirt1 inhibitor CHIC35 (*B*), as indicated, before treatment with 100 nM insulin for the indicated times. AKT phosphorylation at Ser^{473} (*A* and *B*) and Thr^{308} (*A*) and total AKT were determined by immunoblotting. Also shown are Sirt1, Sirt2, GAPDH, and acetylated tubulin levels as control (*B*). *C*, wild type and Sirt2-null mouse embryo fibroblasts were treated with 100 nM insulin for the indicated times and compared for AKT activation. Total AKT and GAPDH served as loading control. *D* and *E*, wild type and stably expressing Sirt2 HeLa cells were treated with 100 nM insulin for the indicated times, and AKT pathway activation was determined by immunoblotting for phospho-AKT and phospho-GSK3β (*D*) or phospho-p70-S6-kinase (*E*), using antibodies against the corresponding AKT phosphorylation sites on these targets. Total AKT, GSK3β, GAPDH, and ERK immunoblots served as control. FLAG immunoblots show Sirt2 expression in the stable cell lines.

trisphosphate. This work identified Sirt1 as the deacetylase responsible for AKT deacetylation. The authors reported direct association of AKT and Sirt1 in HEK-293T cells; however, they have not reported on examining the binding in other cell types. Our results indicate that Sirt2, rather than Sirt1, is the primary interactor and regulator of AKT activation in insulin-responsive cells under normal conditions. Several lines of evidence support this notion. First, we were unable to detect AKT binding to Sirt1 in any insulin-responsive cell line with normal PI3K regulation that we tested, whereas we were able to detect abundant association with Sirt2. Second, Sirt2, but not Sirt1 inhibitors, blocked AKT activation by insulin in insulin-responsive cells. Third, Sirt1 preferentially associated with active AKT, whereas PI3K inhibition dissociated the complex. This mode of regulation suggests that in normal cells with low basal PI3K activity, Sirt1 would not be bound to AKT, thus making its involvement in the AKT activation process unlikely. It is possible, however, that Sirt1 binding is important for stabilizing the active AKT, explaining our observed effects of Sirt1 inhibitors on AKT phosphorylation. Sirt2, on the other hand, appears essential for the AKT activation process even in cells with high PI3K activity, as demonstrated by the PI3K inhibitor washout experiment.

Supporting our findings on the role of Sirt2 in AKT regulation, a recent work reported that nicotinamide phosphoribosyltransferase and Sirt2 can induce AKT deacetylation (43). We were unable, however, to detect AKT acetylation using commercial pan-acetyl-K antibodies in several cell lines, even after AKT purification; hence, we do not have information regarding the effects of Sirt2 on AKT acetylation.

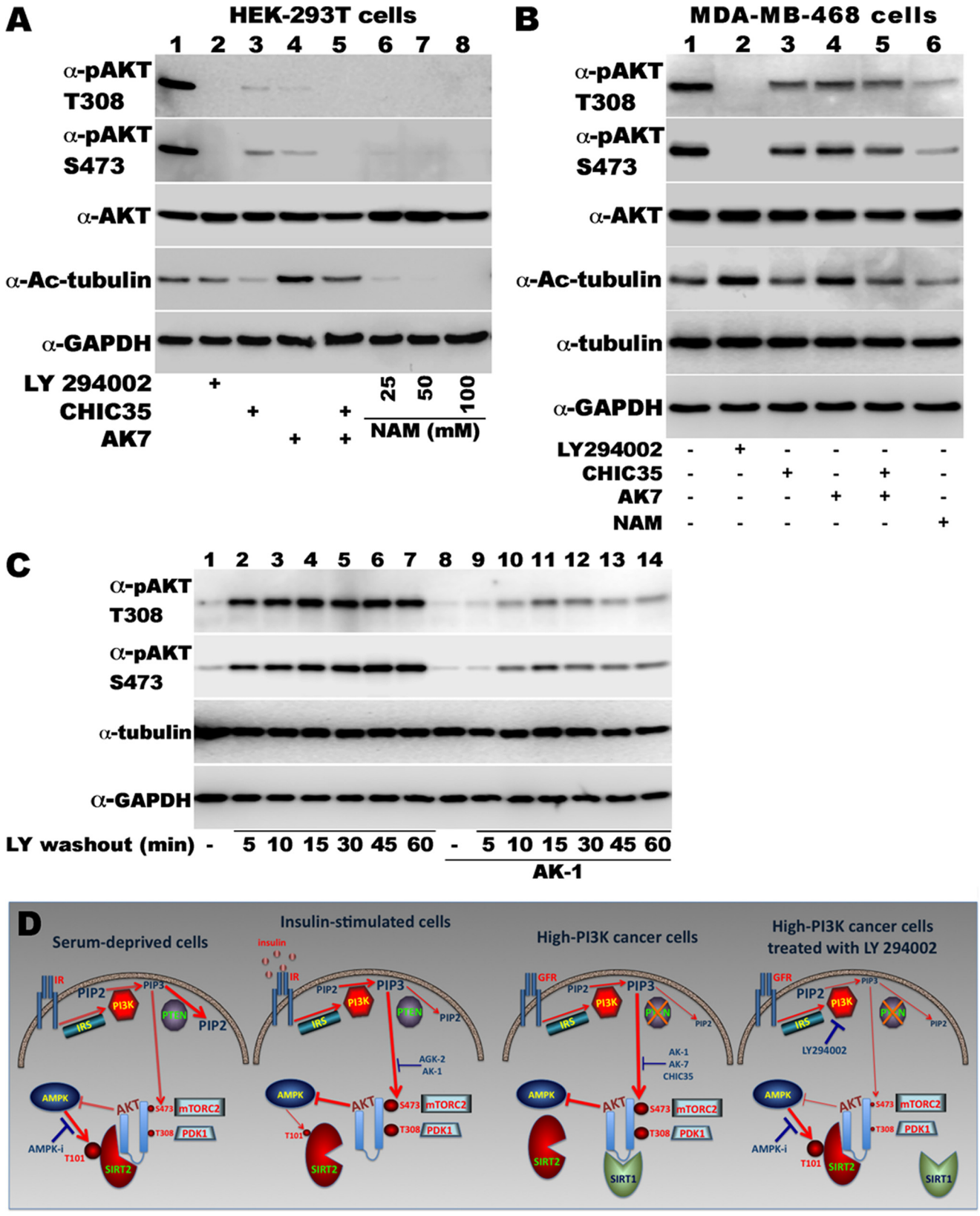

FIGURE 8. **Sirt1 and Sirt2 inhibitors cooperate in reducing AKT phosphorylation in cells with up-regulated PI3K activity.** *A* and *B*, HEK-293T (*A*) and MDA-MB-468 breast cancer cells (*B*) were treated for 2 h with 10 μM LY 294002, 10 μM AK7, 0.5 μM CHIC35, both AK7 and CHIC35, or 25 mM nicotinamide (*NAM*) (*B*) or its indicated concentration (*A*), and AKT phosphorylation was determined by immunoblotting. Total AKT, GAPDH, and acetylated tubulin immunoblots served as control. *C*, HEK-293T cells were treated with 10 μM LY 294002 for 2 h, and where indicated, the drug was removed, and the cells were washed three times with fresh growth medium. The cells were incubated with fresh medium in the presence of vehicle or 10 μM AK1 for the indicated times. Cell extracts were immunoblotted for phospho-AKT. Tubulin and GAPDH were used as loading controls. *D*, a model describing the roles of Sirt1 and Sirt2 in AKT activation in insulin-responsive cells and in cancer cells with up-regulated PI3K activity. Note the changes in AKT phosphorylation levels at the different situations. See "Results" for more details.

Besides the effects of Sirt2 on AKT, our results indicate that the insulin-AKT pathway converges on Sirt2 itself and identify AMPK as a new kinase involved in regulating Sirt2 phosphorylation. So far, very little has been reported on the regulation of Sirt2 activity, especially its regulation by phosphorylation. Sirt2 phosphorylation was detected during mitosis (44), and subsequently CDK-1 was reported to phosphorylate Sirt2 at serine 368 and regulate its function during mitosis (45). Sirt2 Ser^{331} phosphorylation by CDK-2 was reported to inhibit its catalytic activity and affect cell motility (46). Our results demonstrate that Sirt2 is heavily phosphorylated at multiple sites and that AMPK inhibition results in reduced Sirt2 phosphorylation. Thr^{101} was identified as one of the phosphorylation sites that are regulated by AMPK inhibition, although several other sites also showed marked reduction. Our results suggest that Thr^{101} plays a role in the AKT-Sirt2 association. Although Sirt1 has been extensively studied in the past decade, much less work has been done with Sirt2, and only few verified Sirt2 targets and interacting proteins have been established. Thus, the identification of AKT as an insulin-regulated Sirt2 interactor is a significant addition to the field. Identification of the other Sirt2 phosphorylation sites, especially the sites that respond to AMPK inhibition, and determining their role in Sirt2 function could be significant in elucidating Sirt2 regulation.

Both tumor-suppressive and tumor-promoting functions have been attributed to Sirt1 and Sirt2 (31, 47, 48). The common view, supported by knock-out experiments in mice, is that sirtuins can delay tumor formation by maintaining genome integrity (20). On the other hand, cancer cells overexpressing sirtuins could be resistant to DNA-damaging agents and oxidative stress and hold metabolic advantages. Our results demonstrate that Sirt2 and, in certain cases, Sirt1 augment the activation of AKT and its downstream targets. Increased AKT activation is a prominent factor in many human cancers, suggesting that Sirt2 and Sirt1 overexpression could also hold tumor-promoting functions by their effect on AKT activation. In this regard, both Sirt1 and Sirt2 overexpression have been observed in multiple human cancers (31, 48). Our work suggests that whereas Sirt2 activators may be useful in the treatment of metabolic disorders involving insulin resistance, such as type 2 diabetes and obesity, inhibitors of Sirt1 and Sirt2 could be used in cancers with up-regulated PI3K-AKT pathway activation.

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