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SIRT1 negatively regulates the protein stability of HIPK2

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ABSTRACT

In the present study, we investigated whether a histone deacetylase sirtuin 1 (SIRT1) can regulate the protein stability of homeodomain-interacting protein kinase 2 (HIPK2). We observed the evidence of molecular interaction between SIRT1 and HIPK2. Interestingly, overexpression or pharmacological activation of SIRT1 promoted ubiquitination and the proteasomal degradation of HIPK2 whereas inhibition of SIRT1 activity increased the protein level of HIPK2. Furthermore, a SIRT1 activator decreased the level of HIPK2 acetylation whereas an inhibitor increased the acetylation level. These results suggest that SIRT1 may deacetylate and promote the ubiquitination and subsequent proteasomal degradation of HIPK2.

1. Introduction

Homeodomain-interacting protein kinase 2 (HIPK2) is a serine/threonine kinase that regulates DNA damage response and development [1]. HIPK2 functions as a tumor suppressor by activating p53-dependent proapoptotic pathway [2]. Proapoptotic function of HIPK2 was reported to be mediated also by phosphorylation and downregulation of transcriptional corepressor carboxyl-terminal binding protein [3]. Therefore, regulation of HIPK2 activation is of much importance in managing DNA damage response.

Under normal condition, the protein level of HIPK2 is tightly regulated by ubiquitin–proteasomal degradation system [1]. However, HIPK2 escapes from the degradation pathway upon DNA damage and accumulates to aid p53-mediated DNA damage response [4]. The accumulated HIPK2 phosphorylates p53 at serine 46 and this further stabilizes p53 so that p53 transcriptionally activates its downstream genes regulating cell cycle arrest or apoptosis [2]. Therefore, it is important to keep the protein level of HIPK2 low under unstressed conditions or recovering phase following DNA damage response. In unstressed cells, HIPK2 interacts with an E3 ubiquitin ligase, seven in absentia homolog 1 (Siah1). This interaction promotes polyubiquitination and proteasomal degradation of the HIPK2 [5]. The steady state level of HIPK2 protein is also regulated by another E3 ubiquitin ligase WD repeat and SOCS box-containing protein 1 [6]. Siah2 has been reported to promote the degradation of HIPK2 under hypoxic conditions [7].

SIRT1 is a member of mammalian sirtuin family and a class III histone deacetylase (HDAC) [8]. Sirtuins are the mammalian homologues of yeast silent information regulator 2 (Sir2) which has been reported to extend life span of yeast under calorie-restricted conditions [8]. Like Sir2, SIRT1 has drawn much attention as a possible 'longetivity' gene. It has been suggested that SIRT1 is a versatile cytoprotector against diverse cellular stresses. The cytoprotective function of SIRT1 is mediated by deacetylation of histones and nonhistone proteins. The nonhistone substrates of SIRT1 include many important regulators of cell survival, death, and metabolism such as p53, p73, p300, retinoblastoma, and forkhead box class O (FOXO) [9]. via NAD+-dependent deacetylation of these targets, SIRT1 regulates metabolism, aging and many aging-related diseases like cancer and neurodegenerative diseases [9].

Much of antiapoptotic function of SIRT1 is mediated by deacetylation of the tumor suppressor p53 [10]. Deacetylation of p53 by SIRT1 has been shown to decrease transcription-dependent apoptotic and senescence-inducing functions of p53 [10]. Since SIRT1 and HIPK2 play an opposite role in p53 stabilization and activation, we set out to test a possibility that SIRT1 directly antagonizes HIPK2. In the present study, we present evidence that SIRT1 deacetylates HIPK2 and promotes its proteasomal degradation.

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Our study proposes a novel mechanism of HIPK2 protein stability regulation mediated by SIRT1.

2. Materials and methods

2.1. Cell culture and transfection

Human Embryonic Kidney (HEK) 293 cells were grown in Dulbecco's modified Eagle's medium (Welgene, Daegu, Korea) supplemented with 10% heat-inactivated fetal bovine serum and 1% antibiotics plus antimycotics solution (Welgene, Daegu, Korea). Transfection of HEK293 cells with expression vectors encoding SIRT1 or HIPK2 was carried out using Mirus reagent according to the manufacturer's protocol (Mirus Bio, Madison, WI, USA).

2.2. Plasmids

Construction of the expression plasmids for full-length Myc-HIPK2, GFP–HIPK2 and various HIPK2 deletion mutants was described previously [11]. GFP–SIRT1, Flag-SIRT1, and Flag-SIRT1(HY) deacetylase-inactive mutant expression plasmids were kindly provided by Dr. S.J. Um (Sejong University, Korea).

2.3. Antibodies and immunocytochemistry

Antibodies used for the immunoblot and immunostaining were anti-SIRT1 (Millipore, Billerica, MA, USA), anti-HIPK2 (Abcam, Cambridge, UK), anti-α-tubulin (Sigma, St. Louis, MO, USA), anti-Myc, anti-Flag, anti-GFP, anti-HA (abm, Richmond, Canada), anti-ubiquitin (Millipore, Billerica, MA, USA), anti-acetylated lysine (Cell signaling, Danvers, MA, USA). Immunocytochemistry for HEK293 cells was performed as described previously [16].

2.4. Immunoprecipitation and immunoblotting

Immunoprecipitation was performed using 2×10^7 cells in a lysis buffer (20 mM Hepes, pH 7.5, 0.1 M KCl, 0.4 mM EDTA, 0.2% Nonidet P-40, 10 mM β-Mercaptoethanol, 1 μg/ml sodium vanadate, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 0.1 mM PMSF). After incubation at 4 °C on a rotating wheel for 15 min and centrifugation at 13,000 rpm in a benchtop microcentrifuge for 10 min at 4 °C, equal volumes of protein were diluted with lysis buffer lacking NaCl and KCl, then incubated overnight with antibodies. Then protein A Sepharose beads were added (Sigma, St. Louis, MO, USA). After incubation at 4 °C on a rotating wheel for 1 h, the beads were washed three times with lysis buffer. Immunoblotting was performed by conventional methods.

2.5. Reverse transcription polymerase chain reaction

Total RNA was isolated using RNeasy minikit (Qiagen, Valenia, CA). cDNA was synthesized using the Moloney Murine Leukemia Virus Reverse Transcriptase (Promega, Madison, WI, USA) according to the manufacturer's protocol. Reverse transcription (RT) reaction product was used as a template for PCR using the following primer pairs: mouse HIPK2 (forward 5′-GTC ACC ATG ACA CAC CTG CT-3′, reverse 5′-AGG GGG ACA CAC GAT GAG AG-3′), human HIPK2 (forward 5′-CCA CAG CAC ACA CGT CAA ATC-3′, reverse 5′-TTT GCT CTG GTT CAC CGT GTC-3′), β-Actin (forward 5′-CTG GGA CGA CAT GGA GAA-3′, reverse 5′-AAG GAA GGC TGG AAG AGT-3′). Annealing temperature was 58 °C. Reaction products were analysed on 2% agarose gels.

3. Results

3.1. HIPK2 interacted with SIRT1

Previous studies independently reported the presence of SIRT1 and HIPK2 in the promyelocytic leukemia nuclear bodies [12,13]. However, it has not been addressed whether these two enzymes colocalize in the nucleus or interact with each other. To find out if HIPK2 and SIRT1 interact, we first examined their cellular localization by immunocytochemistry. HEK293 cells were transfected with GFP–HIPK2 expression vector and then immunostaining was performed for the endogenous SIRT1. As shown in Fig. 1A, many of the HIPK2-positive nuclear speckles were also positive for the SIRT1, suggesting HIPK2 and SIRT1 colocalized in the nuclear speckles.

We then examined whether HIPK2 and SIRT1 interact *in vivo* by performing coimmunoprecipitation assay. HEK293 cells were transfected with Myc-HIPK2 and Flag-SIRT1 and then the whole cell lysates were immunoprecipitated with anti-Myc, anti-Flag or control IgG. As shown in Fig. 1B, the anti-Myc precipitates contained Flag-SIRT1 but those of control IgG did not, suggesting Myc-HIPK2 interacted with the Flag-SIRT1. The overexpressed Flag-SIRT1 was coimmunoprecipitated with the Myc-HIPK2 (Fig. 1C). In addition, endogenous SIRT1 was also coimmunoprecipitated with the overexpressed Myc-HIPK2 (Fig. 1D).

To examine which domain(s) of the HIPK2 interacted with SIRT1, various deletion mutants of HIPK2 was tested for the coimmunoprecipitation with SIRT1. As shown in Fig. 1E, the interaction domain of HIPK2 (residues 503–860) was coimmunoprecipitated with SIRT1. A deletion mutant spanning the speckle retention signal domain (residues 860–1049) also interacted with SIRT1 to a lesser degree. However, neither N-terminal half containing the kinase domain (residues 1–629) nor the autoinhibitory domain (residues 1049–1189) interacted with SIRT1. These results suggest that HIPK2 interact with SIRT1 mainly via its interaction domain.

3.2. Overexpressed SIRT1 decreased the protein level of HIPK2

SIRT1 is known as a prosurvival molecule against various cellular stressors while HIPK2 is proapoptotic in general [1,9]. Therefore, the interaction of SIRT1 and HIPK2 as we observed in Fig. 1 is likely to cause an antagonistic regulation between these molecules. Since HIPK2 is under a tight regulation of proteasomal degradation [1], we first examined if SIRT1 affects the protein level of HIPK2. To test this possibility, HIPK2 was cotransfected with SIRT1 and the protein level of HIPK2 was examined by immunoblot assay. As shown in Fig. 2A, an increasing amount of SIRT1 resulted in the decrease of HIPK2 protein. In addition, the accumulation of overexpressed HIPK2 over time was suppressed when SIRT1 was cotransfected (Fig. 2B). To find out at which level SIRT1 downregulated the expression of HIPK2, mRNA levels of the exogenous and endogenous HIPK2 were examined by RT-PCR analysis in the presence or absence of SIRT1 overexpression. Unlike protein level, the mRNA levels of both exogenous and endogenous HIPK2 were not altered by SIRT1 overexpression (Fig. 2C). This result suggests that SIRT1 may regulate the expression of HIPK2 at a post-transcriptional level.

3.3. SIRT1 activity was required for the downregulation of HIPK2 protein

To examine if deacetylase activity of SIRT1 is required for the downregulation of HIPK2 protein, the HIPK2-transfected cells were incubated in the presence of a SIRT1 activator, resveratrol. As shown in Fig. 3A, an increasing amount of resveratrol decreased

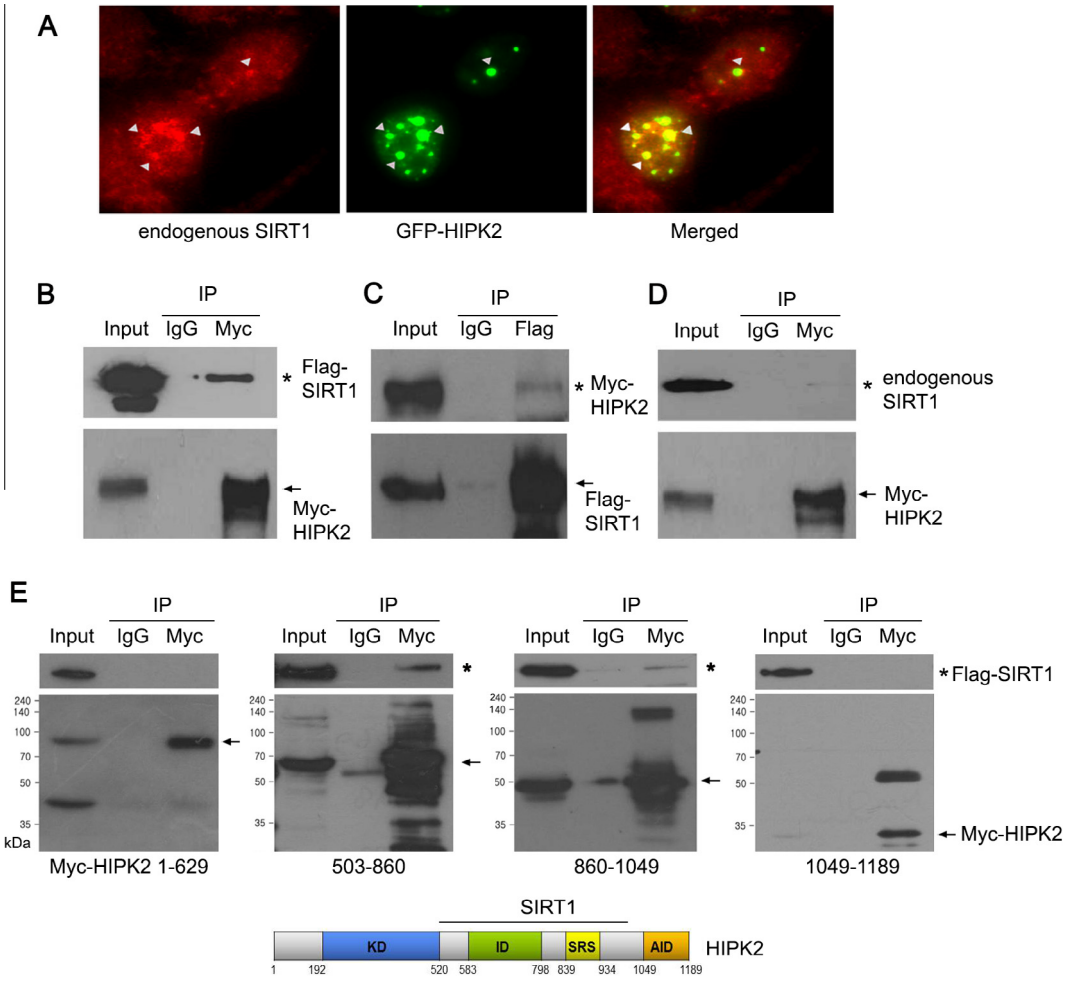

Fig. 1. HIPK2 interacted with SIRT1. (A) HEK293 cells were transfected with GFP–HIPK2 and then immunostained for the endogenous SIRT1 (red fluorescence). Note the colocalization of SIRT1 immunoreactivity and GFP–HIPK2 (arrowheads). (B–D) HEK293 cells were transfected with the indicated expression vectors for SIRT1 and HIPK2 and then immunoprecipitated with anti-Myc (B and D) or anti-Flag (C) antibodies (arrows) and control IgG and then processed for immunoblotting using anti-Flag, anti-Myc or anti-SIRT1 antibodies as indicated (asterisks). (E) HEK293 cells were transfected with various expression vectors for the truncated myc-HIPK2 (amino acids 1–629, 503–860, 860–1049, 1049–1189) and Flag-tagged SIRT1. Each domain of HIPK2 was immunoprecipitated by anti-Myc antibodies (arrows). The blots were also examined for the detection of SIRT1 using anti-Flag antibodies (asterisks). A schematic diagram shows domain structures of HIPK2 and the SIRT1-interacting site. KD, kinase domain; ID, interaction domain; SRS, speckle retention signal; AID, auto-inhibitory domain. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the protein level of HIPK2. The level of GFP cotransfected with HIPK2 as a control was not altered by the resveratrol treatment. In addition, the protein level of HIPK2 gradually decreased as the resveratrol incubation time increased (Fig. 3B). When the protein synthesis was blocked by cycloheximide, the resveratrol-induced decrease of HIPK2 protein level was more evident (Fig. 3C).

Then we examined whether the inhibition of endogenous SIRT1 activity increases the HIPK2 protein level. As shown in Fig. 3D, an increasing dose of a SIRT1 inhibitor, sirtinol, increased the amount of HIPK2 protein but not the cotransfected GFP. To confirm if SIRT1 downregulates HIPK2 protein level via its deacetylase activity, a deacetylase-inactive mutant of SIRT1 (HY mutant) was tested. As shown in Fig. 3E, overexpression of the HY mutant SIRT1 did not change the protein level of HIPK2 whereas the wild type SIRT1 dramatically decreased the HIPK2 protein level. Taken together, these results suggest that SIRT1 downregulated the protein level of HIPK2 via its deacetylase activity.

3.4. SIRT1 promoted the proteasomal degradation of HIPK2

Since we observed the decrease of HIPK2 expression by SIRT1 at a post-transcriptional level, we examined the mechanism of the HIPK2 downregulation. It was previously shown that the HIPK2 protein level can be regulated by either caspase-mediated degradation or proteasomal degradation [1]. To investigate whether the decrease of HIPK2 protein level by SIRT1 is mediated by caspase-dependent proteolysis, we tested if the irreversible broad spectrum caspase inhibitor, zVAD-fmk, can suppress the SIRT1-induced decrease of the HIPK2 protein. As presented in Fig. 4A, zVAD-fmk did not suppress the resveratrol-induced decrease of the HIPK2 protein level, suggesting SIRT1-induced HIPK2 downregulation is not mediated by caspases. Then we examined whether SIRT1 promotes the proteasomal degradation of HIPK2 protein. To test this possibility, we transfected the cells with the expression vectors for HIPK2 with or without SIRT1 and then the cells were incubated with a proteasome inhibitor, MG132. As shown in Fig. 4B, the SIRT1-mediated decrease of the HIPK2 protein was completely suppressed when MG132 was added. In addition, the resveratrol-mediated HIPK2 downregulation was also efficiently blocked by MG132 treatment (Fig. 4C). These results suggest that SIRT1 promoted the proteasomal degradation of HIPK2.

We also found that activation of SIRT1 enhanced polyubiquitination of HIPK2. HEK293 cells were transfected with expression vectors for Myc-HIPK2 and HA-ubiquitin and then incubated with

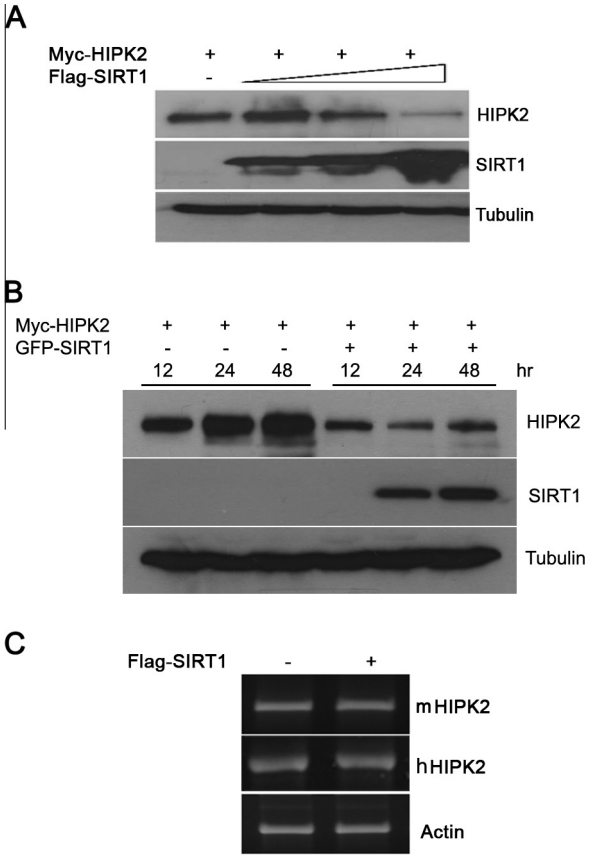

Fig. 2. Overexpressed SIRT1 decreased the protein level of HIPK2. (A) Increasing amounts (0.5, 1 and 2 μg) of SIRT1 expression vectors were transfected into HEK293 cells with Myc-HIPK2 plasmids (1 μg). (B) HIPK2 expression vector was transfected into HEK293 cells with or without GFP–SIRT1 expression vector. After the transfection, the cells were processed for immunoblotting using the listed antibodies at indicated times (A and B). Blots for tubulin served as loading controls. (C) HEK293 cells were transfected with Myc-HIPK2 with or without Flag-SIRT1 cDNA and the mRNA levels of HIPK2 and alpha-actin were determined by RT-PCR at 24 h after the transfection.

resveratrol. Then the cell lysates were immunoprecipitated with anti-HA and probed with anti-Myc antibody. The upwardly shifted bands were much stronger in the resveratrol-treated cells (Fig. 4D), suggesting that the polyubiquitination of HIPK2 was promoted by the resveratrol treatment. Taken together, these results suggest that SIRT1 promoted the polyubiquitination and proteasomal degradation of HIPK2.

3.5. SIRT1 promoted the deacetylation of HIPK2

It has been suggested that acetylation and ubiquitination can occur on the same lysine residues and the deacetylation can lead to ubiquitination on the same residue, promoting the proteasomal degradation of the protein [14]. To examine whether SIRT1 deacetylates HIPK2 to promote its ubiquitination, we first checked whether a SIRT1 activator resveratrol can induce deacetylation of HIPK2. HEK293 cells were transfected with the expression vector for Myc-HIPK2 and then incubated in the presence or absence of resveratrol. Then the cell lysates were immunoprecipitated with anti-acetyl lysine antibody, followed by immunoblotting with anti-Myc antibody to detect the acetylated HIPK2. As shown in Fig. 4E, the amount of acetylated HIPK2 was dramatically reduced when resveratrol was added. This suggests that activation of SIRT1 promoted the deacetylation of the exogenous HIPK2. Then we examined whether inhibition of SIRT1 would increase the acetylation level of HIPK2. As shown in Fig. 4F, nicotineamide, a SIRT1 inhibitor, increased the amount of acetylated HIPK2. These results suggest that SIRT1 can deacetylate HIPK2.

4. Discussion

In the present study, we presented evidence that SIRT1 negatively regulates the protein stability of HIPK2. Our results suggest that deacetylation of HIPK2 by SIRT1 may promote ubiquitination of the deacetylated HIPK2 and the subsequent proteasomal degradation.

HIPK2 has been known to be regulated by various post-translational modifications such as phosphorylation, SUMOylation, and ubiquitination [4]. A recent study by de la Vega and colleagues added acetylation to the list of post-translational modifications on HIPK2 [15]. They reported that HIPK2 is maintained deacetylated by HDAC3 but becomes acetylated by CBP/p300 when the level of reactive oxygen species is elevated [15]. The study suggests that acetylation status determines the subnuclear localization of HIPK2. The deacetylated HIPK2 remains in the nuclear speckles while acetylated forms relocalize to the nucleoplasm, which con-

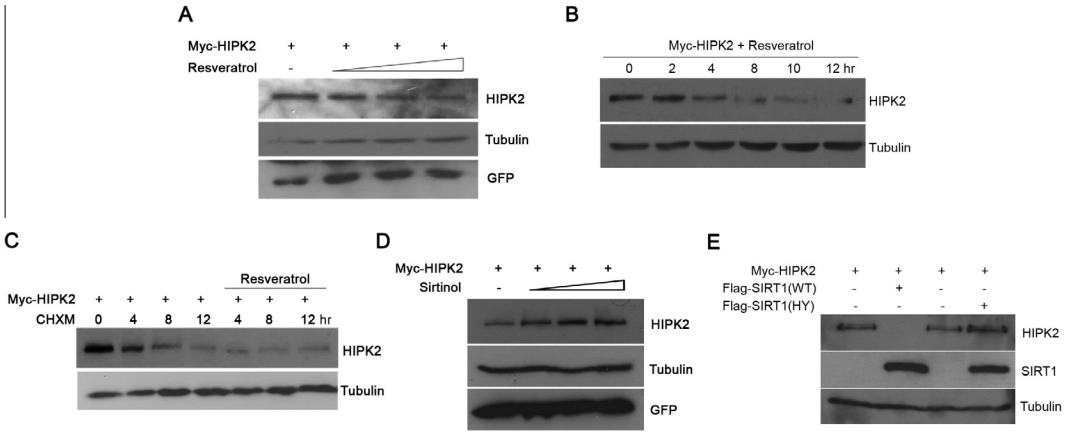

Fig. 3. SIRT1 activity was required for the downregulation of HIPK2 protein. (A and B) HIPK2 expression vector was transfected into HEK293 cells with GFP expression vector as a transfection control. 12 h after the transfection, the cells were treated with an increasing dose (30, 60 and 90 μM) of resveratrol for 12 h (A) or with 50 μM resveratrol for the indicated times (B) and then analyzed by immunoblotting using the indicated antibodies. Blots for tubulin served as loading controls. (C) The changes in the protein level of HIPK2 were monitored following incubation with resveratrol (50 μM) in the presence of cycloheximide (CHXM, 20 μM) at the times indicated. (D) The expression vectors for HIPK2 and GFP were transfected into HEK293 cells. At 12 h after the transfection, the cells were treated with increasing dose of sirtinol (25, 50, and 75 μM) for another 12 h. (E) HEK293 cells were transfected with expression vectors for Myc-HIPK2 and Flag-tagged wild-type or deacetylase domain mutant (HY) SIRT1. Then the level of HIPK2 protein was monitored by immunoblot assay (C–E).

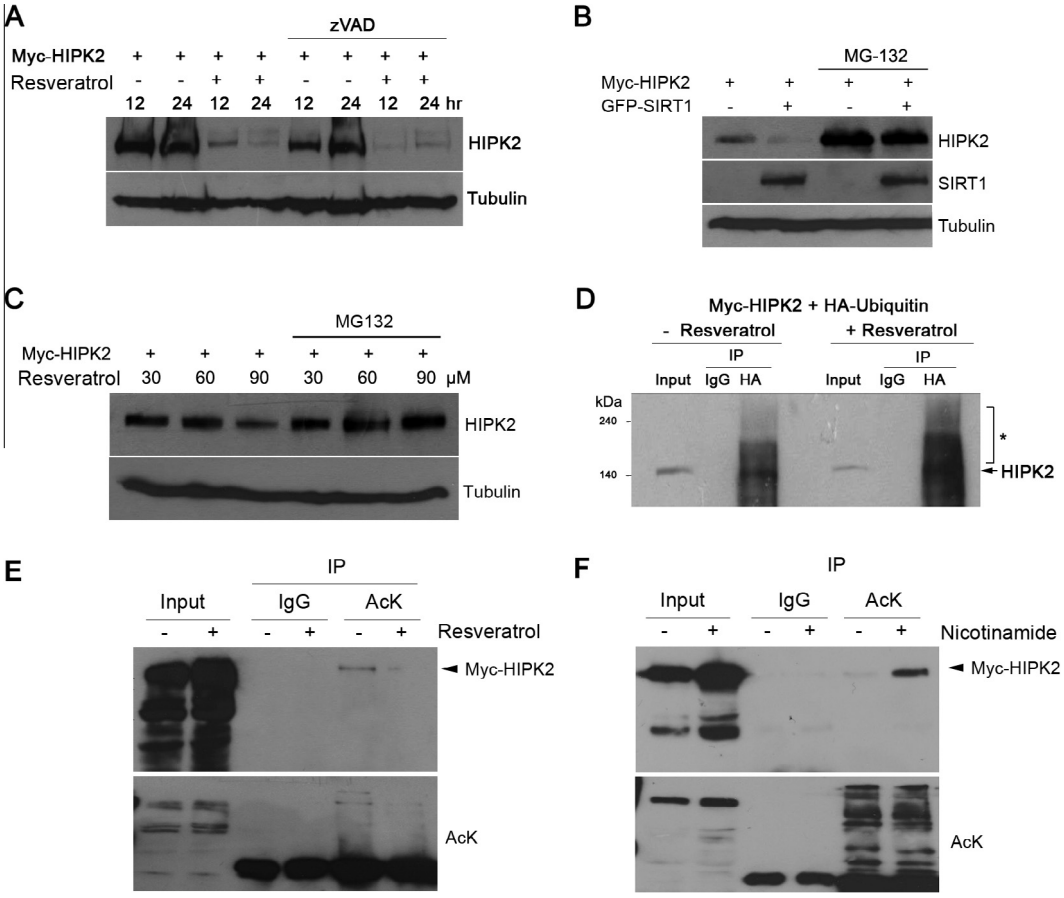

Fig. 4. SIRT1 promoted the proteasomal degradation and deacetylation of HIPK2. (A) HEK293 cells were transfected with Myc-HIPK2 vector and incubated with resveratrol (50 μM) in the presence of zVAD-fmk (25 μM) for the indicated times. (B) Expression vectors for Myc-HIPK2 and GFP–SIRT1 were transfected into HEK293 cells and incubated for 12 h. The cells were then treated with 5 μM of MG132 for another 10 h. (C) HEK293 cells were transfected with Myc-HIPK2 and incubated for 12 h. Then the cells were treated with increasing dose of resveratrol (30, 60 and 90 μM) and MG132 (5 μM) for another 10 h. Then the HIPK2 protein level was monitored by immunoblotting (A–C). Blots for tubulin and GFP served as loading and transfection controls, respectively. (D) HEK293 cells were transfected with plasmids for Myc-HIPK2 and HA-ubiquitin. Then the cells were treated with resveratrol (50 μM) for 12 h in the presence of MG132 (5 μM). Whole cell lysates were immunoprecipitated using anti-HA antibodies or mouse IgG, followed by immunoblotting using anti-Myc. The input lane represents 5% of total cell lysates. Note the upwardly shifted bands in the resveratrol-treated sample (asterisk). To examine if there is a change in the acetylation status of HIPK2 following SIRT1 activation of inhibition, HEK293 cells were transfected with Myc-HIPK2 expression vector in the presence or absence of resveratrol (50 μM) (E) or nicotineamide (10 mM) (F). The cell lysates were immunoprecipitated using anti-acetyl lysine antibody or control IgG. Then the precipitates were examined by immunoblot assay using anti-Myc antibody to detect the acetylated Myc-HIPK2.

trols the survival threshold to oxidative stress [15]. The present study elucidates a different aspect of deacetylation mediated by SIRT1, namely, the regulation of protein stability of HIPK2. Our data suggest that SIRT1 deacetylates HIPK2, which promotes ubiquitination and the proteasomal degradation of HIPK2. Although it remains to be determined whether the lysine residues deacetylated by SIRT1 are the direct target of ubiquitination, it is possible that SIRT1 and HIPK2 ubiquitin ligase compete for the same lysine residues. Examples of the competition between acetylation and ubiquitination on lysine residues are not rare [14]. Forkhead box class O3, Forkhead box P3, and LKB1 have been shown to be deacetylated by SIRT1, which facilitates ubiquitination and proteasomal degradation of the deacetylated proteins [16–18]. Thus, SIRT1 can carry out its function by regulating proteasomal degradation of its deacetylation target proteins.

SIRT1 is a nuclear deacetylase whose substrates include important regulators of cell survival, differentiation and metabolism [8]. Especially SIRT1 functions as cytoprotector by deacetyling critical regulators of cell survival or death. For example, SIRT1 deacetylates the tumor suppressor p53 to inhibit its transcriptional activity, resulting in the suppression of apoptosis induced by various genotoxic stresses [10]. In response to severe DNA damage, HIPK2 forms a complex with p53 and phosphorylates it at serine residue 46, which activates its proapoptotic transcriptional activity [2]. In addition, there have been independent studies suggesting SIRT1 promotes survival whereas HIPK2 promotes cell death during oxidative stress [15,19]. Therefore, our data suggesting SIRT1 can suppress HIPK2 function by promoting its proteasomal degradation provide a novel insight into the cell survival-death regulation mechanism modulated by SIRT1 during cellular stress.

Previous studies reported that HIPK2 binds to CBP [12] and becomes acetylated by CBP/p300 [15]. de la Vega et al. suggested that the acetylated HIPK2 is relocalized from the nuclear speckles to the nucleoplasm [15]. However, they did not examine whether acetylation of HIPK2 by CBP prevents ubiquitination and the proteasomal degradation. Since the protein level and the acetylation level of HIPK2 increased following the incubation with a SIRT1 inhibitor, it is possible that the acetylated HIPK2 is protected from the ubiquitin–proteasomal degradation. It remains to be studied whether CBP/p300 or other acetyl transferases stabilize the HIPK2 protein by acetylation.

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