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Cbl regulates the activity of SIRT2

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ABSTRACT

SIRT2 is a member of the sirtuin family of NAD^+-dependent protein deacetylases. It is involved in metabolic homeostasis and has been linked to the progression of age-related diseases. Casitas B-lineage lymphoma (Cbl) proteins regulate signal transduction through many pathways and, consequently, regulate cell function and development. Cbl proteins are ubiquitin ligases that ubiquitinate and target many signaling molecules for degradation. The function of SIRT2 is modulated by post-translational modifications. However, the precise molecular signaling mechanism of SIRT2 through interactions with Cbl proteins has not yet been established. In this study, we investigated the potential regulation of SIRT2 function by the Cbl mammalian family members Cbl-b and c-Cbl. We found that Cbl-b and c-Cbl increased the protein level and stability of SIRT2 and that Cbl-b and c-Cbl interact with SIRT2. They were also found to regulate the deacetylase activity of SIRT2. Further investigation revealed that Cbl-mediated SIRT2 regulation occurred via ubiquitination of SIRT2.

1. Introduction

Sirtuins regulate important biological pathways in bacteria and eukaryotes. Sirtuins have been shown to influence a wide range of cellular processes like aging, transcription, apoptosis, and inflammation. Sirtuin enzymes belong to the family of class III NAD^+-dependent deacetylases that are conserved from bacteria to humans. In mammals, seven sirtuins (SIRT1–SIRT7), which share a similar catalytic core, have been identified [1,2]. SIRT proteins affect metabolism and are often considered potential targets for pharmacologic intervention due to the importance of their function [3]. Recent studies have shown that SIRT1 can deacetylate non-histone proteins, including various transcription factors involved in growth regulation, stress responses, and endocrine signaling. It can also suppress p53-dependent apoptosis induced by DNA damage [4,5]. In contrast, SIRT2 functions are less well-known, compared to those of SIRT1. In the recent report, we found that the activity of SRIT2 are regulated by Src [6]. Src associates with E3 ubiquitin ligase Cbl [7–12]. So we investigate whether E3 ubiquitin ligase Cbl can regulate the activity of SIRT2.

SIRT2 overexpression delays cell cycle progression [13]. SIRT2 is known to deacetylate lysine 40 of α-tubulin and to colocalize with HDAC6 [14]. SIRT2 has also been shown to regulate adipocyte differentiation through FOXO1 acetylation/deacetylation [5]. Inhibition of SIRT2 can control cell fate and tumorigenesis by regulating the acetylation status of several tumor suppressors including p53 and FOXO1 [15]. SIRT2 interacts with 14-3-3 β/γ proteins and presents a novel negative regulatory mechanism for p53 [16]. SIRT inhibitors have been shown to induce cell death and p53 acetylation by targeting both SIRT1 and SIRT2 [17]. SIRT2 deacetylates lysine residues in the catalytic domain of p300, a histone acetyltransferase, which maintains its active form via autoacetylation [18]. Acetylation of SIRT2 by p300 attenuates its deacetylase activity [19].

Cbl (Casitas B-lineage lymphoma) proteins act as cytoplasmic adaptors. Cbl-b and c-Cbl may negatively regulate signaling by acting as E3 ubiquitin ligases, resulting in the proteasomal degradation of activated molecules [20,21]. Three members of the Cbl protein family, c-Cbl, Cbl-b, and Cbl-3, have been extensively studied in mammals [22]. Cbl-b and c-Cbl are members of the mammalian Cbl family and share an overall domain structure with a high degree of sequence identity, particularly in the N-terminal half (84% identity). Cbl-b and c-Cbl proteins consist of the following domains: an N-terminal tyrosine kinase-binding (TKB) domain, which mediates the binding to specific phospho-tyrosine motifs; a Zn-coordinating RING finger domain that interacts with E2 ubiquitin-conjugating enzyme; a proline-rich region that contains functional SH3 domain-binding sites; and a C-terminal leucine zipper-like region, with high homology to ubiquitin-associated (UBA)

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domains [20,23,24]. Interestingly, Cbl-b and c-Cbl, but not Cbl-3, have a UBA domain at their C-terminal ends [20]. The RING finger has been associated with the negative regulation of a number of tyrosine kinases, including epidermal growth factor receptor and Syk [20].

The regulation of gene expression can be controlled by regulating transcription factors, which are tightly regulated by post-translational modifications, such as phosphorylation, acetylation, and ubiquitination [25]. Cyclin-dependent kinase 1 phosphorylates SIRT2, leading to mitotic regulation, and Erk1/2 and Src also regulate SIRT2 activity via phosphorylation [6,26,27]. However, the effects of post-translational modification, i.e., ubiquitination, on the molecular signaling mechanisms of SIRT2 by the Cbl family of RING-type E3 ligases have not yet been characterized.

In this study, we found that Cbl-b and c-Cbl increased the protein level and stability of SIRT2. Cbl-b and c-Cbl interact with and reduce the ubiquitination of SIRT2. The deacetylase activity of SIRT2 increased upon the activation of Cbl-b and c-Cbl. Cbl proteins play a role in the regulation of SIRT2 function. These results provide evidence that the deacetylase activity of SIRT2 is regulated through its ubiquitination by Cbl family proteins.

2. Materials and methods

2.1. Plasmids, antibodies, and reagents

Myc-tagged wild-type SIRT2 and HA-tagged p53 were constructed in a CMV promoter-derived mammalian expression vector (pCS4+). Plasmids for FLAG-tagged Ubiquitin, HA-tagged Cbl-b, HA-tagged c-Cbl, a truncation mutant in which the N-terminal SH2 domain deletion of Cbl-b (ΔN) as indicated, and a ubiquitin ligase-deficient RING finger of c-Cbl (ΔC3AHN) as indicated were generously provided by Dr. N. Kim (Chonnam National University, Korea) [28]. For knockdown of murine *c-Cbl*, oligonucleotides targeting a 19-base sequence (5′-GGAGACACTTTCCGGATTA-3′) were synthesized. Sense and antisense oligonucleotides were annealed and ligated into pSuper-retro (Oligoengine). The following antibodies were used: HA (12CA5) and Myc (9E10) from Roche Applied Science; Cbl-b (G-1), Flag (M2), α-Tubulin (B-5-1-2), and acetylated-tubulin from Sigma–Aldrich; c-Cbl (17/c-Cbl) from BD Biosciences; and SIRT2 (H-95) from Santa Cruz Biotechnology. Chemicals MG132 from Calbiochem and cycloheximide (CHX) from Sigma–Aldrich were used.

2.2. Cell culture and transient transfection

HEK 293 and MCF-7 cell lines were cultured at 37 °C and 5% CO_2 in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 100 g/mL streptomycin. DMEM, FBS and antibiotics were purchased from Gibco. Transient transfection was performed using polyethyleneimine (Polysciences, Inc.). Total amounts of transfected plasmids in each group were equalized by the addition of an empty vector.

2.3. Protein stability assay

HEK 293 cells were co-transfected with Myc-tagged SIRT2, HA-tagged Cbl-b, and HA-tagged c-Cbl plasmids. Media were refreshed after 24 h. Transfected cells were incubated for the indicated time points, then treated with 40 μM cycloheximide (CHX), and harvested with lysis buffer, as described below. The protein levels were analyzed by immunoblotting using the anti-Myc antibody.

2.4. Immunoblotting and Immunoprecipitation

For immunoblotting, transfected HEK 293 cells were harvested after washing with ice-cold PBS and lysed in ice-cold lysis buffer [25 mM HEPES (pH 7.5), 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 10% glycerol, 25 mM NaF, 1 mM EDTA, 1 mM Na_3VO_4, 250 μM PMSF, 10 μg/mL leupeptin, and 10 μg/mL aprotinin]. After centrifugation, supernatants containing 30 μg of total protein were subjected to SDS–PAGE, and then, the proteins were transferred to a PVDF membrane. For immunoprecipitation, the supernatants of the cell lysates were incubated with appropriate antibodies and protein A or protein G-agarose beads. The lysate supernatants or immunoprecipitated proteins were resolved by SDS–PAGE and transferred to PVDF membranes. Proteins were visualized using the appropriate primary antibodies and horseradish peroxidase-coupled secondary antibodies (Amersham Biosciences) by immunoblotting, and the membranes were developed with enhanced chemiluminescence lighting (ECL) solution (Amersham Biosciences). Signals were detected and analyzed with an LAS4000 luminescent image analyzer (Fuji Photo Film Co., Tokyo, Japan).

2.5. Luciferase reporter assay

MCF-7 Cells were seeded in 24-well plates the day before transfection. Cells were transfected with CMV promoter-driven β-galactosidase reporter (pCMV-β-gal), p53 luciferase reporter (p53-Luc) and the indicated combinations of expression plasmids. After 36 h, luciferase activities were measured using the Luciferase Reporter Assay Kit (Promega) using a luminometer, and the activities were normalized to corresponding β-galactosidase activities for transfection efficiency. Experiments were performed in triplicate and repeated at least three times. The averages and standard deviations (S.D.) of representative experiments are shown.

2.6. In vitro tubulin deacetylation assay

For the tubulin deacetylation assay, HEK 293 cells transfected with Myc-SIRT2, HA-tagged Cbl-b, and HA-tagged c-Cbl were harvested after washing with ice-cold PBS and then lysed in ice-cold lysis buffer [25 mM HEPES (pH 7.5), 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 10% glycerol, 25 mM NaF, 1 mM EDTA, 1 mM Na_3VO_4, 250 μM PMSF, 10 μg/mL leupeptin, and 10 μg/mL aprotinin]. Supernatants containing 30 μg of total protein were subjected to SDS–PAGE after centrifugation. The proteins were transferred to a PVDF membrane, and acetylated tubulin was visualized by western blotting using antisera specific for acetylated tubulin [29].

2.7. Statistical analysis

All experiments were performed with three independent replicate samples and were repeated at least twice to give qualitatively identical results. Results are expressed as mean ± standard deviations for luciferase assays. Data were analyzed using the Student's *t*-test, and $p < 0.05$ was considered significant.

3. Results

3.1. Cbl-b and c-Cbl regulate the protein levels of SIRT2

Cbl-b and c-Cbl were determined to be ubiquitin ligases. The Cbl ubiquitin ligases were identified as critical negative regulators. In order to understand the mechanism of SIRT2, we decided to examine whether the RING-type E3 ubiquitin ligases Cbl-b and

c-Cbl were able to affect the protein levels of SIRT2. Experimentation showed the endogenous protein levels of SIRT2 were increased with increasing amounts Cbl-b and c-Cbl, in a dose dependent manner (Fig. 1A and B, top panel). In addition, we showed that the exogenous protein levels of SIRT2 were increased with increasing amounts Cbl-b and c-Cbl, in a dose dependent manner (Fig. 1C and D, top panel). The Cbl-b ΔN mutant lacks the N-terminal SH2 domain rendering it ubiquitin ligase-deficient, and c-Cbl ΔC3AHN has a deletion in the RING finger domain rendering it ubiquitin ligase-deficient. Cbl-b ΔN failed to increase SIRT2 protein levels as seen with wild-type Cbl-b (Fig. 1E), and c-Cbl ΔC3AHN produced similar results (Fig. 1F). Furthermore, knockdown of *c-Cbl* (*sh-c-Cbl*) attenuated the increase in SIRT2 protein levels observed with wild-type c-Cbl. These results suggest that Cbl proteins regulate the protein levels of SIRT2.

3.2. Cbl-b and c-Cbl enhance the protein stability of SIRT2

Since Cbl-b and c-Cbl may regulate the expression of SIRT2 at the level of transcription, translation, or protein stability, the influence of Cbl-b and c-Cbl on the stability of SIRT2 was examined using the translation inhibitor cycloheximide (CHX) for the indicated time points to identify the molecular mechanism of the Cbl family-induced increase in SIRT2 protein levels.

In the absence of Cbl-b and c-Cbl, SIRT2 protein degradation had a half-life of about 8 h. However, Cbl-b and c-Cbl significantly enhanced the stabilization of SIRT2 and prolonged the half-life of the SIRT2 protein (Fig. 2A, top panel). These results suggest that Cbl-b and c-Cbl regulate SIRT protein levels by affecting stability.

3.3. Cbl-b and c-Cbl interact with and reduce the ubiquitination of SIRT2

Protein stability can be regulated through post-translational modifications by several kinases. We suggested above that SIRT2 protein levels and stability were increased by Cbl-b and c-Cbl (Figs. 1 and 2). A major pathway in the regulation of protein levels is mediated by the E3 ubiquitin ligase and ubiquitin system [30]. We took the following approaches to determine whether Cbl-b and c-Cbl affect the ubiquitination of SIRT2. First, the possible interaction between Cbl family and SIRT2 was examined by immunoprecipitation assays of the expressed proteins in HEK 293 cells. Results showed that endogenous SIRT2 bound to both overexpressed Cbl-b and c-Cbl (Fig. 3A, top panel). As the ubiquitin–proteasome pathway is a major system for post-translational modification of protein levels [30], we next investigated whether Cbl-b and c-Cbl had an effect on the ubiquitin-mediated degradation of SIRT2. SIRT2 showed decreased poly-ubiquitination and increased protein levels when coexpressed in HEK 293 cells with Cbl-b and c-Cbl, in the absence of the proteasome inhibitor MG132 (Fig. 3B, left panels). In contrast, SIRT2 protein levels were not affected by Cbl-b and c-Cbl when the cells were treated with

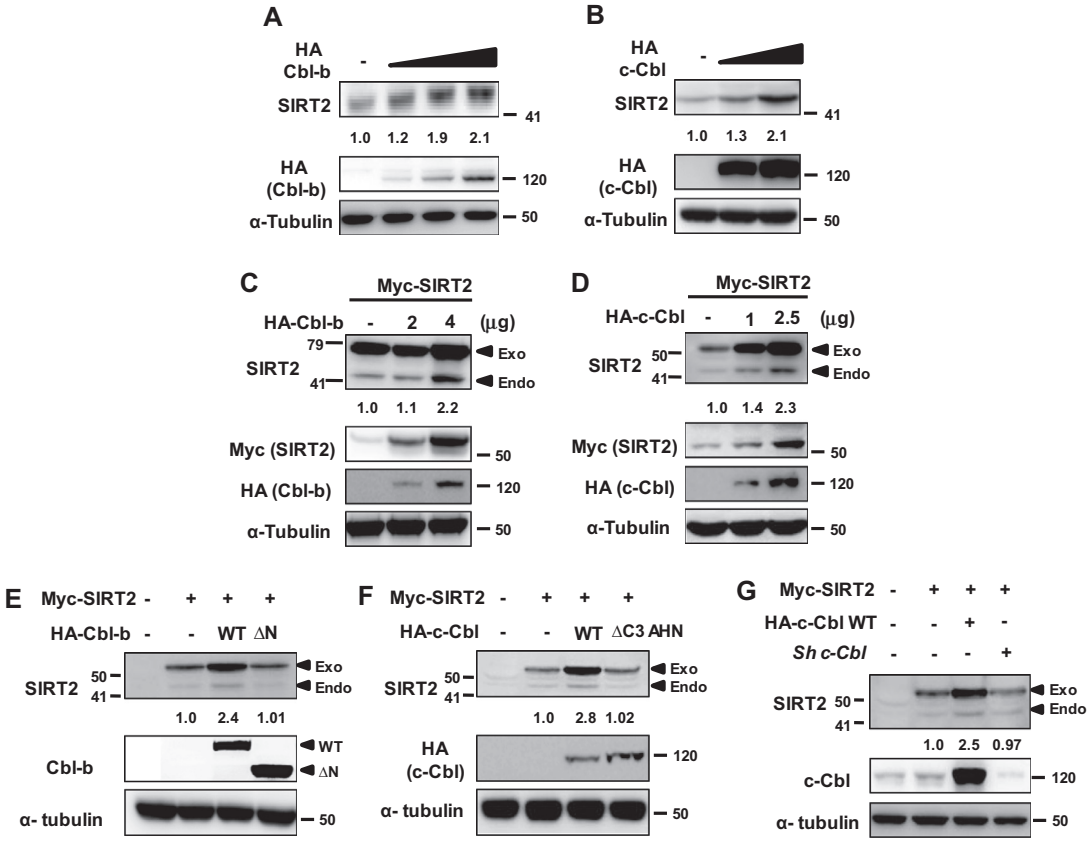

Fig. 1. The expression levels of SIRT2 are regulated by Cbl-b and c-Cbl. (A and B) For measurement of endogenous protein levels in response to increased amounts of Cbl-b and c-Cbl, HEK 293 cells were transfected with HA-tagged Cbl-b, HA-tagged c-Cbl, or a control vector plasmid. Protein levels of SIRT2 were determined by immunoblotting using an anti-SIRT2 antibody. Tubulin was used as a loading control. (C and D) For measurement of the exogenous protein levels in response to increased amounts of Cbl-b and c-Cbl, HEK 293 cells were transfected with Myc-tagged SIRT2, HA-tagged Cbl-b, HA-tagged c-Cbl, or a control vector plasmid. Protein levels of SIRT2 were determined by immunoblotting using anti-SIRT2 and anti-Myc antibodies. Tubulin was used as a loading control. (E, F, and G) HEK 293 cells were transfected with Myc-tagged SIRT2, HA-tagged Cbl-b, HA-tagged c-Cbl, an N-terminal SH2 domain deletion mutant of Cbl-b (ΔN), or a ubiquitin-deficient RING finger mutant of c-Cbl (ΔC3AHN). Additionally, cells were transfected with shRNA against murine *c-Cbl* (*sh-c-Cbl*) or a control vector plasmid. Protein levels of SIRT2 were determined by immunoblotting using an anti-SIRT2 antibody. Tubulin was used as a loading control. 'Exo' indicates transfected exogenous SIRT2 protein, and 'Endo' indicates endogenous SIRT2 protein.

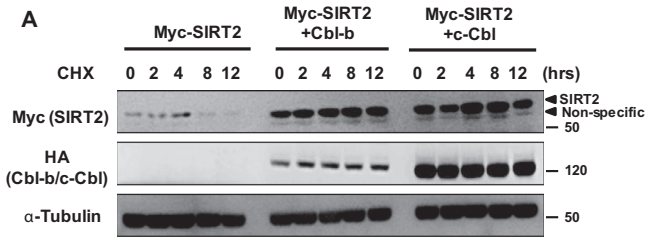

Fig. 2. The protein stability of SIRT2 is enhanced by Cbl-b and c-Cbl. (A) HEK 293 cells were co-transfected with Myc-tagged SIRT2, HA-tagged Cbl-b, and HA-tagged c-Cbl, and the cells were treated with cycloheximide (CHX, 40 μM). After 24 h, transfected cells were harvested at the indicated time points. Protein levels of SIRT2 were determined by immunoblotting using an anti-Myc antibody.

MG132 (Fig. 3B, right panels). These results indicate that the Cbl family regulates the ubiquitin-mediated proteasomal degradation of SIRT2.

3.4. Cbl-b and c-Cbl regulate the deacetylase activity of SIRT2 and down-regulate p53

In previous reports, SIRT2 was shown to deacetylate and down-regulate the transcriptional activity of p53 [19]. We examined whether Cbl-b and c-Cbl could affect the deacetylase activity of SIRT2. The deacetylase activity of SIRT2 increased with increased Cbl-b or c-Cbl (Fig. 4A and B, top panel). Also, the Cbl-b ΔN mutant lacks the N-terminal SH2 domain, rendering it ubiquitin ligase-deficient, and c-Cbl ΔC3AHN has a deletion in the RING finger domain, rendering it ubiquitin ligase-deficient. Cbl-b ΔN significantly attenuated the enhancement of SIRT2 deacetylase activity seen with wild-type Cbl-b (Fig. 4C), and c-Cbl ΔC3AHN yielded similar results (Fig. 4D). Furthermore, knockdown of *c-Cbl* (*sh-c-Cbl*) attenuated the deacetylase activity of SIRT2, compared to wild-type c-Cbl (Fig. 4E). In a previous report, SIRT inhibitors were shown to induce p53 acetylation by targeting SIRT2 [17]. We decided to examine whether the E3 ubiquitin ligases Cbl-b and c-Cbl could affect p53 transcriptional activity via regulation of SIRT2. SIRT2-mediated down regulation of p53 transcriptional activity was attenuated by Cbl-b and c-Cbl, likely because of the increase in endogenous SIRT2 activity (Fig. 4F). These results suggest that Cbl family proteins affect SIRT2 deacetylase activity and affect p53 transcriptional activity by regulating SIRT2 protein levels.

4. Discussion

Several studies suggest that SIRT2 (NAD-dependent deacetylase sirtuin-2) promotes chromatin structure, transcriptional silencing, DNA repair, and control of cellular life span, cell cycle, cell death, and aging-related disease.

In the present study, we found evidence that the RING-type E3 ubiquitin ligase Cbl family plays a role in the regulation of SIRT2 function. Cbl-b and c-Cbl may affect the protein stability and transcriptional activity of SIRT2 through post-translational modifications such as ubiquitination. Our results indicate that Cbl-b and c-Cbl activation positively regulates the protein levels and stability of SIRT2 through ubiquitination in the proteasomal degradation pathway (Figs. 1–3). Cbl-b ΔN and c-Cbl ΔC3AHN significantly attenuated the enhancement of SIRT2 deacetylase activity observed with wild-type Cbl (Fig. 4). Cell death, cell cycle, and p53 acetylation have been shown to be regulated by SIRT2 [17,31,32]. Moreover, the deacetylase activity of SIRT2 was also shown to be enhanced by Cbl-b and c-Cbl activities. Our results suggest that Cbl-b and c-Cbl act as positive regulators of SIRT2. Studies of the Cbl family will enhance our understanding of the regulatory mechanisms of SIRT2.

Mammals have seven homologs of SIRT1–7, which affect aging and metabolism, and are potential targets for pharmacologic intervention. Sirtuin activity has been associated with many human diseases, which commonly occur later in life such as cancer and diabetes as well as cardiovascular and neurodegenerative diseases. SIRT2 levels are increased in a number of tumors. SIRT2 has received much attention due to its possible impact on longevity, while important biological and therapeutic roles of other sirtuins have been underestimated and just recently recognized. SIRT2

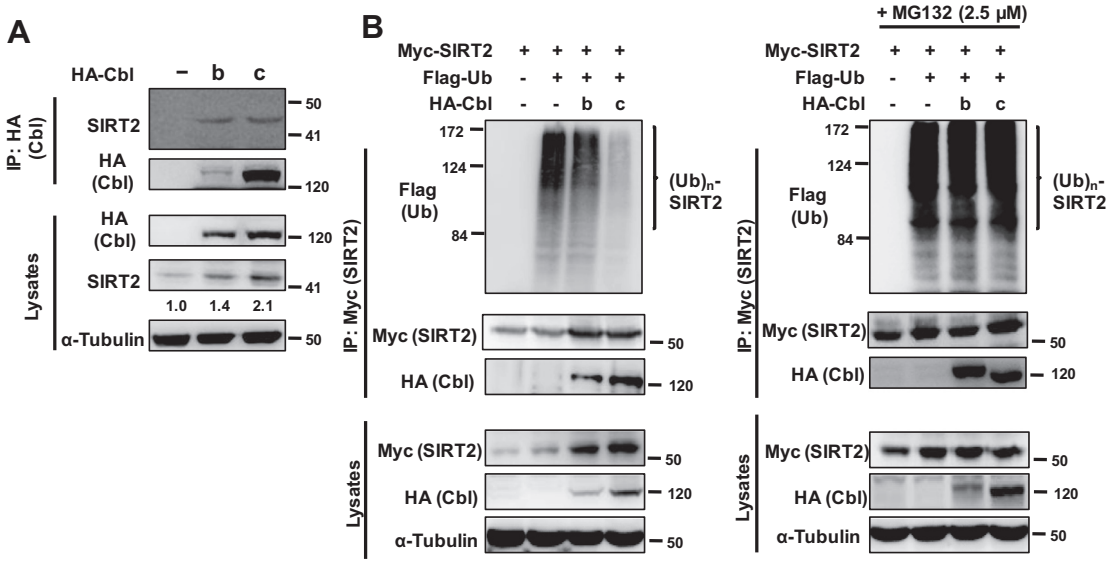

Fig. 3. Cbl-b and c-Cbl decrease the ubiquitination of SIRT2. (A) HEK 293 cells were transfected with HA-Cbl-b or HA-c-Cbl. The interaction between endogenous SIRT2 and overexpressed Cbl proteins was analyzed after immunoprecipitation with an antibody for Cbl [IP: HA (Cbl)] followed by immunoblotting with an anti-SIRT2 antibody (top panels). 'b' indicates transfected Cbl-b, and 'c' indicates transfected c-Cbl. (B) HEK 293 cells were transfected with the indicated combinations of Myc-tagged SIRT2, FLAG-tagged ubiquitin, HA-tagged Cbl-b, or HA-tagged-c-Cbl. Cells were then treated with the proteasome inhibitor MG132 (2.5 μM) or DMSO for 18 h. The protein levels of SIRT2 in cell lysates were compared by immunoblotting. Ubiquitination of SIRT2 was analyzed after immunoprecipitation with an antibody for SIRT2 [IP: Myc (SIRT2)] followed by immunoblotting with an anti-ubiquitin antibody [Flag (Ub)]. 'b' indicates transfected Cbl-b, and 'c' indicates transfected c-Cbl.

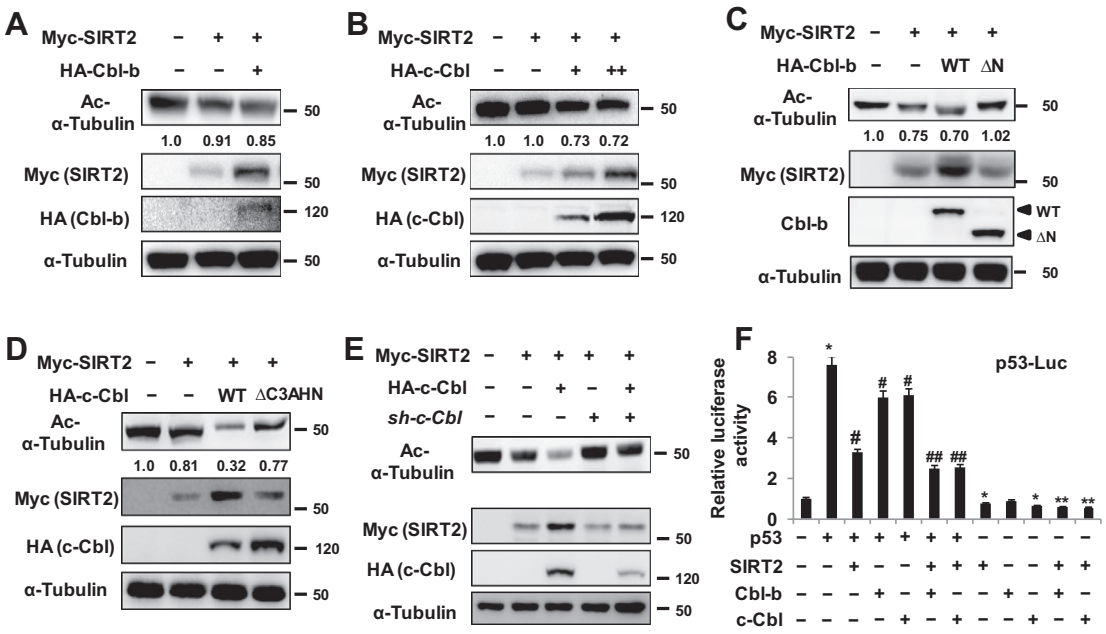

Fig. 4. Deacetylase activity of SIRT2-mediated downregulation of p53 is augmented by Cbl-b and c-Cbl. (A and B) HEK 293 cells were transfected with the indicated combinations of Myc-tagged SIRT2, HA-tagged Cbl-b, HA-tagged-c-Cbl, or a control vector. Acetylated tubulin was visualized by western blotting using antisera specific for acetylated tubulin (Ac-α-tubulin). The levels of α-tubulin were used as loading controls. (C, D, and E) HEK 293 cells were transfected with Myc-tagged SIRT2, HA-tagged Cbl-b, HA-tagged c-Cbl, an N-terminal SH2 domain deletion mutant of Cbl-b (ΔN), or a ubiquitin-deficient RING finger mutant of c-Cbl (ΔC3AHN). Additionally, cells were transfected with shRNA against murine *c-Cbl* (*sh-c-Cbl*) or a control vector plasmid. Acetylated Tubulin was visualized by western blotting using antisera specific for acetylated tubulin (Ac-α-tubulin). The levels of α-tubulin were used as loading controls. (F) MCF-7 cells were transfected with p53-luc (0.3 μg), pCMV-β-gal (0.05 μg), and indicated combinations of p53 (0.3 μg), SIRT2 (0.3 μg), and Cbl family proteins (Cbl-b and c-Cbl; 0.5 μg each). Luciferase activities were measured, and averages and standard deviations (SD) of triplicate measurements are shown. * and ** indicate a significant difference, compared to control or SIRT2 transfection alone, respectively ($p < 0.05$, by *t*-test). # and ## indicate a significant difference, compared to p53 alone or a cotransfection with p53 and SIRT2, respectively ($p < 0.05$, by *t*-test).

overexpression reduces cell proliferation and regulates cell death in response to DNA damage. The use of cancer cell lines suggests that SIRT2 is a tumor suppressor gene [33]. These studies will be important to elucidate the role of SIRT2 activation in specific human cancers.

P53 is crucial in multicellular organisms, where it regulates the cell cycle and, thus, functions as a tumor suppressor, preventing cancer. In particular, involvement in the downregulation of p53 tumor suppressor activity highlights the sirtuins as oncogenes. Acetylation has been shown to augment p53 DNA binding and to stimulate p53-mediated transactivation of its downstream target genes through the recruitment of co-activators. P53 can also be deacetylated by HDAC1 and SIRT1 [34]. HDAC6 and SIRT2 regulate the acetylation state, thereby possibly participating in the regulation of cell cycle progression [35,36].

Additionally, Cbl-mediated ubiquitination may be a potential target for therapeutic intervention. Because the ubiquitination/degradation system controls many cellular processes, targeting proteins involved in this mechanism may provide therapeutic clues in pathological conditions. Therapeutically, ectopic c-Cbl expression in osteosarcoma cells could reduce the expression of EGFR and PDGFR-α, resulting in decreased cell proliferation and survival and reduced lung metastasis in mice. Thus, promoting receptor tyrosine kinase ubiquitination and degradation by targeting c-Cbl may prove to be efficient at reducing tumor growth and metastasis in cancers in which receptor tyrosine kinase expression is excessive [37]. Our studies on this may provide a valid therapeutic approach, especially when combined with standard therapies.

In conclusion, our results indicate that RING-type E3 ubiquitin ligases in the Cbl family have important roles in SIRT2 signaling mechanisms by enhancing stability and deacetylase activity.

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