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# SIRT1 suppresses cellular accumulation of β-TrCP E3 ligase via protein degradation

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ABSTRACT

β-Transducin repeat-containing protein (β-TrCP), an E3 ligase, promotes the degradation of substrate proteins in response to various stimuli. Even though several β-TrCP substrates have been identified to date, limited information of its upstream regulators is available. Here, we showed that SIRT1 suppresses β-TrCP protein synthesis via post-translational degradation. SIRT1 depletion led to a significant increase in the β-TrCP accumulation without affecting the mRNA level. Consistently, β-TrCP protein accumulation induced by resveratrol was further enhanced upon SIRT1 depletion. Rescue of SIRT1 reversed the effect of resveratrol, leading to reduced β-TrCP protein levels. Proteasomal inhibition led to recovery of β-TrCP in cells with SIRT1 overexpression. Notably, the recovered β-TrCP colocalized mostly with SIRT1. Thus, SIRT1 acts as a negative regulator of β-TrCP synthesis via promoting protein degradation.



## 1. Introduction

The ubiquitin–proteasome proteolytic pathway regulates the degradation of proteins that are not necessary for control of cellular action and physiological activity. The E3 ubiquitin ligase complex plays a central role in catalyzing the poly-ubiquitination of substrate proteins in the proteasomal degradation pathway [1]. β-Transducin repeat-containing protein (β-TrCP, also termed Fbw1 or FWD1) is an F-box protein of the SCF E3 ubiquitin ligase complex that is highly conserved across species from yeast to human [1,2]. β-TrCP has a broad range of substrate proteins, including β-catenin, IkB, cdc25A, ATF4, and WEE1A [3], which contain a common destruction motif, DSG (X)2+nS, that provides the recognition site for interactions with the WD40 repeat domain of β-TrCP [2,3]. Phosphorylation of the DSG motif is the principal biochemical reaction for β-TrCP-mediated recognition and destruction of substrate proteins [1–3]. Thus, the cellular activity or level of β-TrCP determines the fate of substrate proteins and subsequent cellular responses against extra- or intracellular stimuli.

The cellular level of β-TrCP is endogenously low, but upregulated by the action of signaling pathways, such as Wnt/β-catenin [4–6] and JNK [7]. Oncogenic BRAF-mediated activation of the MAPK pathway contributes to the maintenance of high β-TrCP expression in human cancer [8]. In addition to these signaling pathways, extracellular stimuli, including glucose deprivation, energy restriction, and reactive oxygen species, promote β-TrCP expression [9–11]. Recently, β-TrCP upregulation by resveratrol was reported [11]. Resveratrol serves as an activator of SIRT1, an $NAD^+$-dependent deacetylase that regulates the function of substrate proteins by deacetylating lysine residues [12]. Therefore, diverse cellular actions triggered by resveratrol are mediated via activation of SIRT1 [12,13]. However, resveratrol has additionally been shown to control cellular physiology in a SIRT1-independent manner [14–21]. Upon DNA damage, SIRT1 controls apoptosis, repair, and cell cycle progression via deacetylation of several substrate proteins, such as p53 [22], Ku70 [23], and Nbs1 [24]. SIRT1 additionally mediates the DNA damage response through interactions with the tumor suppressor, HIC1 [25], or metabolic enzymes, including GAPDH [26]. SIRT1 is present in both the cytosol and nucleus [27]. In response to cytotoxic stimuli, the protein not only translocates to the nucleus [28] but also controls nuclear translocation of cytosolic GAPDH [26].

In the present study, we investigated the effect of SIRT1 on the cellular level of β-TrCP. Our results collectively show that SIRT1 inhibits β-TrCP accumulation by promoting protein degradation, providing evidence for a novel mechanism underlying the regulation of β-TrCP protein stability.

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## 2. Materials and methods

### 2.1. Cell culture and reagents

MDA231, MDA453 and 293T cells were cultured in Dulbecco's Modified Eagle's medium (Cat. No. LM001-05, Welgene, Korea). HCT116, MCF7, SW480, H358 and H1299 cells were cultured in RPMI (Cat. No. LM011-01, Welgene, Korea), and HeLa cells in Minimum Essential medium (Cat. No. LM007-07, Welgene, Korea). All media were supplemented with 10% fetal bovine serum (Cat. No. 43640, JRS, Woodland, CA, USA) and 1% penicillin/streptomycin (Cat. No. 15140, GIBCO, CA, USA). Resveratrol (Cat. No. R5010) and MG132 (Cat. No. C2211) were purchased from Sigma (St. Louis, MO, USA). Cells were cultured at 37 °C under a humidified atmosphere containing 5% (v/v) $CO_2$.

### 2.2. Gene silencing via RNA interference

For transient silencing experiments, cells were transfected with SIRT1-specific or control siRNAs using Lipofectamine RNAi MAX reagent (Cat. No. 13778-150, Invitrogen, CA, USA), according to the manufacturer's instructions. Negative control siRNA was purchased from Invitrogen (Cat. No. 12935-300). The oligonucleotide sequences of SIRT1 siRNAs were as follows: SIRT1-siRNA #1, 5′-ACUUUGCUGUAACCCUGUA-3′, and SIRT1-siRNA #2, 5′-ACAGUUUCAUAGAGCCAUG-3′. SIRT1-depleted cell lines in which SIRT1 or control sh-RNA were stably incorporated were prepared as described previously [25].

### 2.3. Western blot analysis

Cellular protein levels were determined using Western blot analysis. Briefly, cells were lysed in TNN buffer (120 mM NaCl, 40 mM Tris–HCl, pH 8.0, 0.5% NP-40, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 100 mM sodium fluoride and 1 μg/ml each of leupeptin, aprotinin and pepstatin). Protein samples were boiled in SDS sample buffer, separated using a SDS–polyacrylamide gel, electrophoretically transferred to nitrocellulose membranes (Cat. No. 10401396, Whatman, Maidstone, UK), and probed with antibodies against β-TrCP (Cat. No. sc-33213, Santa Cruz Biotechnology, CA, USA), SIRT1 (Cat. No. sc-55404, Santa Cruz Biotechnology) or β-actin (Cat. No. sc-47778, Santa Cruz Biotechnology). The chemiluminescent detection kit (Cat. No. RPN 2106, GE Healthcare, Little Chalfont, UK) was used to visualize protein–antibody complexes.

### 2.4. Total RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted from cells using the RNeasy Mini kit (Cat. No. 74106, Qiagen, GmBH, Germany) according to the manufacturer's protocol. For cDNA synthesis, total RNA was reverse-transcribed using the iScript cDNA synthesis kit (Cat. No. #170-8890, Bio-Rad). Reverse transcriptase-polymerase chain reaction was performed using the Maxime PCR PreMix kit (i-StarTaq) (Cat. No. #25167, Intron Biotechnology, Korea). The following primer sequences for semi-quantitative RT-PCR were used: β-TrCP forward primer (5′-CAA GAG AAG GCA CTC AAG TTT-3′) and reverse primer (5′-TCC ACA AAT TCC ACT TGA TCT-3′); SIRT1 forward primer (5′-CAA ACT TTG CTG TAA CCC TGT-3′) and reverse primer (5′-CAG CCA CTG AAG TTC TTT CAT-3′); β-actin forward primer (5′-ACC ACA CCT TCT ACA ATG AGC-3′) and reverse primer (5′-CTT CAT GAT GGA GTT GAA GGT-3′).

### 2.5. Real-time RT-PCR analysis

Real-time PCR was performed using KAPA SYBR FAST Universal qPCR kit (Kapa Biosystems, Woburn, MA, USA) and CFX96 real-time PCR detection system (BioRad). The primer sequences used for real-time RT-PCR were: SIRT1 forward primer (5′-CAG CCA CTG AAG TTC TTT CAT-3′) and reverse primer (5′-CAA ACT TTG CTG TAA CCC TGT-3′); β-TrCP forward primer (5′-CAG ATG AGG ATT GTG TCA TCA-3′) and reverse primer (5′-TAG TGA GTG GCT CAT CTG ACA-3′); β2-microglobulin forward primer (5′-AAG GAC TGG TCT TTC TAT CTC TTG TA-3′) and reverse primer (5′-ACT ATC TTG GGC TGT GAC AAA GTC-3′); TATA-box binding protein forward primer (5′-CAG CAG CAG CAG CAG CAA C-3′) and reverse primer (5′-GCT GCC ACT GCC TGT TGC-3′). Thermal cycle reaction was as follows: initial cycle at 95 °C for 3 min, which was then followed by 40 cycles at 95 °C for 15 s, 60 °C for 20 s, and 72 °C for 30 s. Each RT-PCR reaction was repeated at least three times to know reproducibility. β2-microglobulin and TATA-box binding proteins were used as internal controls for the normalization of each sample. Each normalized value was determined by subtracting the mean threshold cycle (Ct) of β2-microglobulin and TATA-box binding proteins from the Ct values of β-TrCP and SIRT1, yielding ΔCt numbers. The relative expression was calculated by using a comparative threshold cycle ($2^{-\Delta\Delta C(t)}$) method [29].

### 2.6. Immunofluorescence

Cells grown on glass coverslips were fixed with 100% (v/v) cold methanol on ice for 5 min and blocked with 1% (v/w) bovine serum albumin solution for 1 h at room temperature. Next, cells were stained with β-TrCP antibody in 1% (v/w) bovine serum solution for 2 h and washed three times with phosphate-buffered saline. To visualize β-TrCP and SIRT1 proteins, cells were incubated with Alexa Fluor 488 (Cat. No. A11055, Invitrogen) and Alexa Fluor 555-conjugated secondary antibodies (Cat. No. A21430, Invitrogen) for 1 h at room temperature, respectively. Nuclei were stained using Vectashield mounting medium containing DAPI (Cat. No. H-1200, Vector Laboratories, CA). Stained cells were viewed under a laser scanning confocal microscope (Cat. No. LSM 710, Carl Zeiss, Oberkochen, Germany).

## 3. Results

### 3.1. SIRT1 suppresses cellular accumulation of β-TrCP

To determine whether SIRT1 regulates cellular β-TrCP, we initially determined the β-TrCP protein level after depletion of SIRT1. Data obtained using established HeLa cell lines stably depleted of endogenous SIRT1 via introduction of SIRT1 shRNA [26] revealed an increase in β-TrCP protein (Fig. 1A). All three clonal populations (clones #1, #7, and #9) depleted of SIRT1 exhibited higher β-TrCP levels than the clone transfected with sh-control plasmid. In addition to the established clonal population, we observed an increase in β-TrCP in cells transiently depleted of SIRT1 via transfection with specific siRNA (Fig. 1B). Under conditions where two different SIRT1 siRNAs were transfected, increased β-TrCP synthesis was consistently observed, irrespective of the siRNA used. This SIRT1 siRNA-induced increase in β-TrCP was observed in three other cell lines, specifically, MDA231, SW480 and HCT116 (Fig. 1C), implying that SIRT1 regulation of β-TrCP is not cell type-specific. Our findings collectively indicate that SIRT1 suppresses cellular accumulation of β-TrCP. Consistently, introduction of SIRT1 led to a decrease in the cellular β-TrCP level (Fig. 1D), further confirming SIRT1-mediated suppression of β-TrCP accumulation.

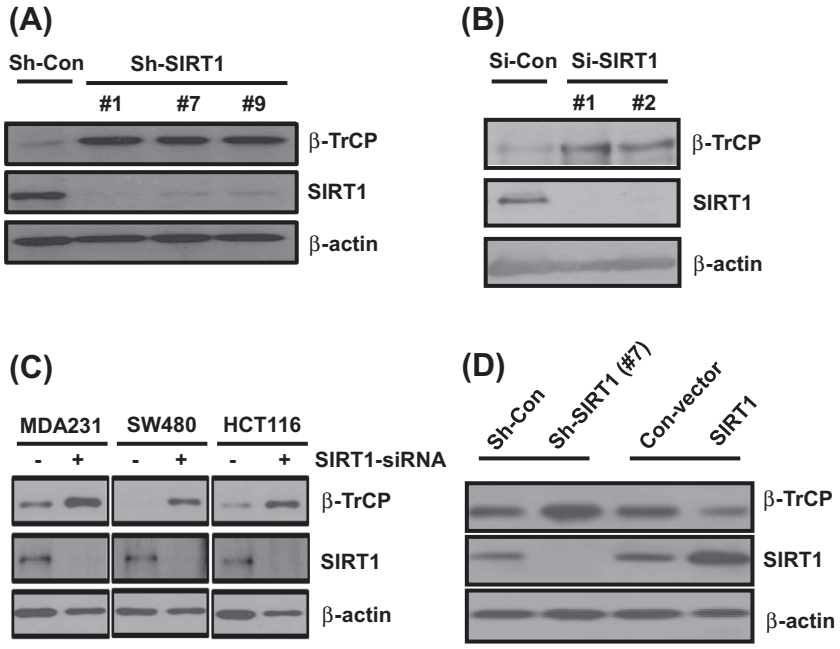


**Fig. 1.** SIRT1 suppresses β-TrCP accumulation. (A) β-TrCP accumulation upon SIRT1 depletion. β-TrCP and SIRT1 protein levels were determined in HeLa cell clones #1, #7, and #9 established after the introduction of sh-SIRT1. sh-Con indicates HeLa cells transfected with the sh-control vector. (B and C) Levels of β-TrCP and SIRT1 proteins were determined in (B) HeLa cells, and (C) MDA231, SW480, and HCT116 cells transiently transfected with either SIRT1 siRNA (+) or control siRNA (−). #1 and #2 indicate SIRT1-siRNA #1 and #2. (D) Suppression of β-TrCP by SIRT1. β-TrCP and SIRT1 protein levels were determined in HeLa cells expressing SIRT1 and empty vector (Con-vector).

### *3.2. SIRT1 suppresses β-TrCP accumulation induced by resveratrol, glucose deprivation or excess pyruvate*

To further evaluate SIRT1 inhibition of β-TrCP accumulation, we examined the effect of SIRT1 under conditions of β-TrCP induction. The cellular level of β-TrCP is considerably enhanced in LNCaP cells treated with resveratrol [11]. The precise effects of resveratrol on SIRT1 are controversial, since resveratrol-mediated physiological events may either be dependent [12–14] or independent of SIRT1[14–21]. Consistent with previous reports, resveratrol treatment led to accumulation of β-TrCP in diverse cell lines, including HeLa, MDA231 and SW480 (Fig. 2A). However, in contrast to a prior report that β-TrCP accumulation by resveratrol is positively regulated by SIRT1 [11], our experiments showed that SIRT1 negatively affects resveratrol-induced accumulation of β-TrCP. SIRT1 depletion further enhanced β-TrCP protein accumulation induced by resveratrol in HeLa cells (Fig. 2B). The negative action of SIRT1 on β-TrCP accumulation was consistently observed in SW480 and HT1080, the two other cell lines examined. Both SW480 and HT1080 cells similarly exhibited SIRT1 depletion-mediated enhancement of β-TrCP induction by resveratrol (Fig. 2C). Thus, SIRT1 prevents β-TrCP accumulation, both in the absence and presence of resveratrol stimulation. Consistently, rescue of SIRT1 expression in SIRT1-depleted HeLa cell clones, #1 and #7, via exogenous introduction reduced β-TrCP accumulation induced by resveratrol (Fig. 2D). In contrast to prior findings [11], our data indicate that the resveratrol-stimulated β-TrCP level is not positively regulated by SIRT1. Rather, SIRT1 suppresses β-TrCP accumulation induced by resveratrol.

To further confirm these findings, we employed other stress conditions to induce β-TrCP accumulation, including glucose deprivation and excess pyruvate (Supplementary Fig. 1). Glucose deprivation has been shown to enhance β-TrCP levels [9–11]. Under conditions of glucose elimination or pyruvate addition, SIRT1 depletion additionally enhanced β-TrCP accumulation (Supplementary Fig. 1A and B). We conclude that SIRT1 suppresses β-TrCP accumulation induced by glucose deprivation and pyruvate, similar to the situation with resveratrol.

### *3.3. SIRT1 suppression of β-TrCP accumulation is not mediated by transcriptional regulation*

The cellular level of β-TrCP is controlled via either transcriptional activation or post-translational degradation [4]. To evaluate the mode of β-TrCP accumulation, we initially examined β-TrCP mRNA levels under conditions of SIRT1 depletion through semi-quantitative RT-PCR. While the β-TrCP protein was increased, mRNA levels remained unaffected by SIRT1 depletion (Fig. 3A and B). Specifically, at 12–48 h after the addition of SIRT1 siRNA, the β-TrCP mRNA level was maintained in HeLa and HCT116 cells (Fig. 3A). Consistently, the β-TrCP mRNA level remained unchanged despite an increase in β-TrCP protein following SIRT1 depletion in two other cell lines, MDA231 and 293T (Fig. 3B), indicating that β-TrCP is not regulated at the transcriptional level. Additionally, the resveratrol-induced increase in β-TrCP protein was not accompanied by a concomitant increase in β-TrCP mRNA (Fig. 3C). To further validate our present finding, we reanalyzed the β-TrCP mRNA levels through the quantitative real-time RT-PCR analysis. As shown in the results obtained from the analysis of semi-quantitative RT-PCR, real-time RT-PCR also exhibited that β-TrCP mRNA levels were not altered, either when SIRT1 was depleted by SIRT1-siRNA in HeLa, HCT116, and 293T cells (left panel, Fig. 3D), or β-TrCP protein synthesis was increased in SW480 and HCT116 cells by resveratrol (right panel, Fig. 3D). These findings indicate that resveratrol-induced accumulation of β-TrCP is not attributed to control of transcription. Furthermore, SIRT1 regulation of cellular β-TrCP accumulation occurs at the post-transcriptional level.

### *3.4. SIRT1 suppression of β-TrCP accumulation is regulated by post-translational degradation of β-TrCP protein*

To further establish whether SIRT1 suppression of β-TrCP accumulation is mediated post-translationally, cells were treated with MG132, an inhibitor of 26S proteosomal degradation. MG132 treatment led to accumulation of β-TrCP protein even in the absence of SIRT1-siRNA and resveratrol (Fig. 4A), indicating degrada-

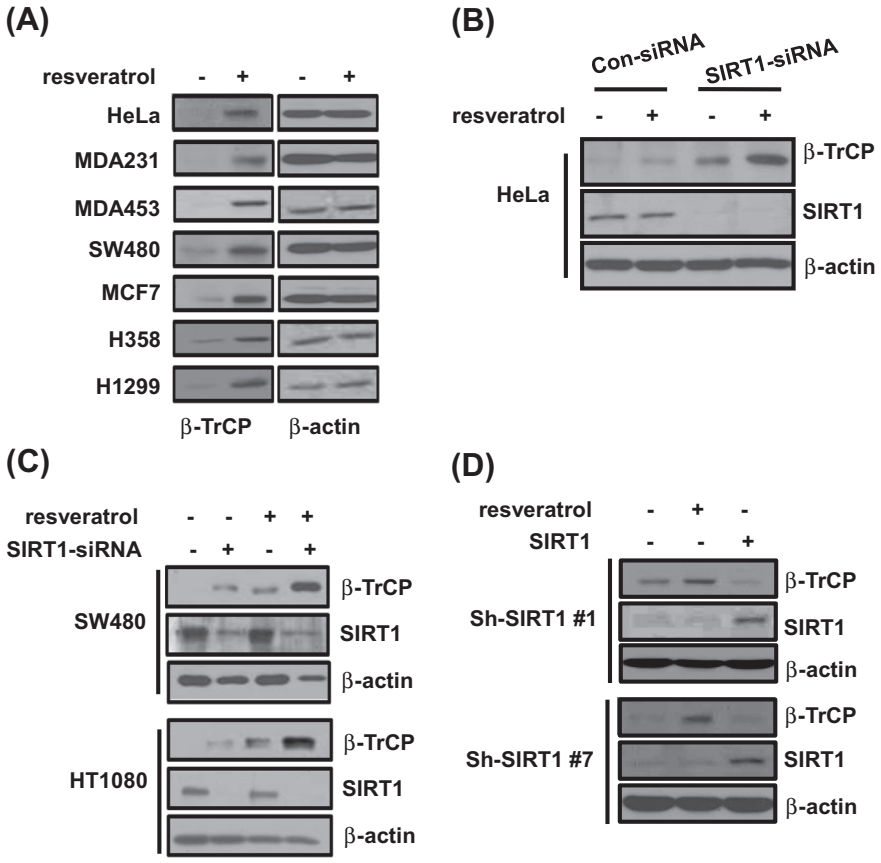


**Fig. 2.** SIRT1 suppresses resveratrol-induced accumulation of β-TrCP. β-TrCP and SIRT1 levels were determined in the following cultures treated with resveratrol. (A) β-TrCP accumulation in diverse cell lines subjected to resveratrol treatment. Resveratrol (100 μM) was added to cultures of human cancer cell lines, including HeLa, MDA231, MDA453, SW480, MCF7, H358, and H1299. (B and C) Enhancement of resveratrol-induced β-TrCP accumulation upon SIRT1 depletion. Resveratrol (100 μM) was added to cultures of HeLa (B), and SW480 and HT1080 cells (C) pretreated with SIRT1-siRNA. (D) Reduction of resveratrol-induced β-TrCP following SIRT1 rescue. SIRT1-depleted HeLa clones #1 and #7 were transfected with SIRT1 (+) and empty vector (−). SIRT1-rescued and control HeLa cells were treated with resveratrol (100 μM).

tion of β-TrCP protein when the cellular level of SIRT1 is not reduced (and thus maintained). In addition, MG132-mediated recovery of β-TrCP protein was observed in both the cytosolic and nuclear fractions (Fig. 4B). Immunofluorescence analysis was performed to compare β-TrCP recovery between HeLa cells with and without SIRT1 overexpression, with a view to establishing whether this protein recovery is associated with SIRT1. In the absence of MG132, less β-TrCP fluorescence was observed in the nucleus than cytosol (Fig. 4C and Supplementary Fig. 2), as evident from Western blot analysis (Fig. 4B). As anticipated, MG132 treatment led to an increase in β-TrCP fluorescence, irrespective of SIRT1 overexpression. However, the recovery patterns of β-TrCP were different. In cells without SIRT1 overexpression (transfected with empty vector), β-TrCP fluorescence was evident in both the cytosol and nucleus, whereas in those overexpressing SIRT1, β-TrCP fluorescence was predominantly observed in the nucleus, indicating that β-TrCP was recovered mostly in the nucleus when SIRT1 was overexpressed. Under SIRT1 overexpression condition, recovered β-TrCP colocalized with SIRT1 in the nucleus. Our findings indicate that SIRT1 suppression of β-TrCP accumulation is mediated via promotion of protein degradation.

## 4. Discussion

Considerable attention has focused on defining the substrate proteins of β-TrCP using the recognition motif of plausible substrates. These studies have resulted in the identification of several substrate proteins in association with physiological changes. However, limited information is available on the upstream regulators and underlying regulatory mechanisms.

Here, we conclude that SIRT1 functions as a negative regulator of β-TrCP protein synthesis, based on several lines of evidence. Transient SIRT1 depletion with siRNA led to significant expression of β-TrCP protein. Increased β-TrCP synthesis was consistently observed in established HeLa cells depleted of endogenous SIRT1 via incorporation of sh-SIRT1 plasmid. Upon SIRT1 rescue, the β-TrCP level reverted back to that observed in cells without SIRT1 depletion. Our findings are inconsistent with previous reports showing that β-TrCP expression is increased as a consequence of transient induction of SIRT1 [11]. This proposal was based on several observations: (1) SIRT1 induction occurred prior to the β-TrCP increase, (2) induction of β-TrCP by OSU-CG12 was not observed in cells transfected with dominant-negative SIRT1, and (3) overexpression of SIRT1 enhanced the level of β-TrCP. In contrast to earlier findings, we showed that SIRT1 overexpression does not lead to an increase, but rather, a decrease in the β-TrCP protein level. This result was in keeping with data obtained with SIRT1-depleted cells that showed an increase in β-TrCP. Our finding that SIRT1 depletion increases β-TrCP synthesis was not cell type-specific, and observed in various cell lines, including HeLa, HCT116, 293T, SW480, and MDA231. Accordingly, we propose that SIRT1 functions as a negative regulator of β-TrCP synthesis. At present, we cannot explain the discrepancy between our results on SIRT1 regulation of β-TrCP expression and the two earlier reports. In the figures of prior report, however, we could observe a significant decrease in SIRT1 when the β-TrCP level was increased, as we presented here.

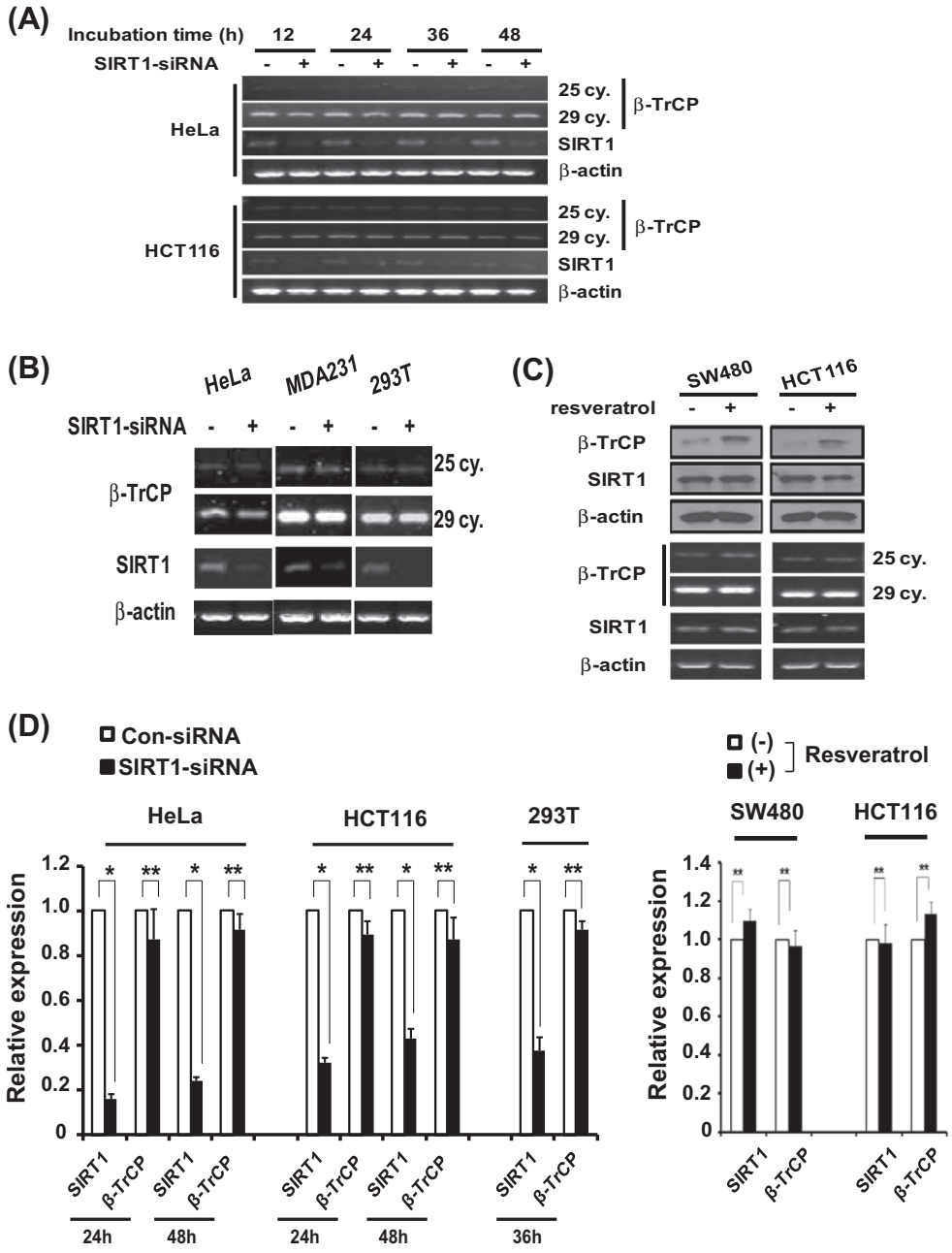


**Fig. 3.** SIRT1 suppression of β-TrCP accumulation is controlled at the post-transcriptional level. (A and B) Levels of β-TrCP and SIRT1 mRNAs were measured using semi-quantitative RT-PCR in (A) HeLa, HCT116, MDA231 and 293T cells transfected with SIRT1- (+) or control- siRNA (−). (C) SW480 and HCT116 cells were treated with 100 μM resveratrol. β-Actin was used as the internal control. Cy indicates the cycle for amplification of β-TrCP mRNA via semiquantitative RT-PCR. (D) The mRNA levels of β-TrCP and SIRT1 were analyzed through real-time RT-PCR, in HeLa, HCT116 and 293T cells treated with (+) and without SIRT1-siRNA (−) (left panel), and in SW480 and HCT116 cells treated with (+) and without resveratrol (−) (right panel). The relative levels of SIRT1 and β-TrCP mRNAs in cells with SIRT1-siRNA or with resveratrol were determined, based on those in cells with control-siRNA or without resveratrol, respectively. The mean value of β2-microglobulin and TATA-binding protein expression was used for the normalization of SIRT1 and β-TrCP mRNA levels in each sample. $^{*}p < 0.001$, $^{**}p > 0.05$.

The basal level of β-TrCP is low, but upregulated upon development of stress. Data from an experiment performed under conditions where the cellular β-TrCP level was increased prior to SIRT1 depletion support SIRT1 suppression of β-TrCP synthesis. β-TrCP synthesis induced by resveratrol was further enhanced upon SIRT1 depletion. A recent study showed that that SIRT1 is inactivated at cytotoxic concentrations of resveratrol [16]. At the resveratrol concentration leading to β-TrCP induction, we also observed cell death, which was more severe upon SIRT1 depletion. Thus, resveratrol-mediated increase in β-TrCP occurred, not due to activation, but rather inactivation of SIRT1 activity. Indeed, response to resveratrol may either be dependent [12,13] or independent of SIRT1 [14–21]. As shown in resveratrol, SIRT1 inactivation also leads to an increase of cell death in response to anticancer agents [28,30]. Actually, SIRT1 is important for telomere maintenance [31]. Similar to SIRT1 inactivation, loss of telomere maintenance attenuates cellular survival against anticancer agents [32].

The cellular β-TrCP level is controlled at various steps of transcription and post-translational degradation. β-TrCP mRNA expression is increased by activation of signaling pathways involving Wnt

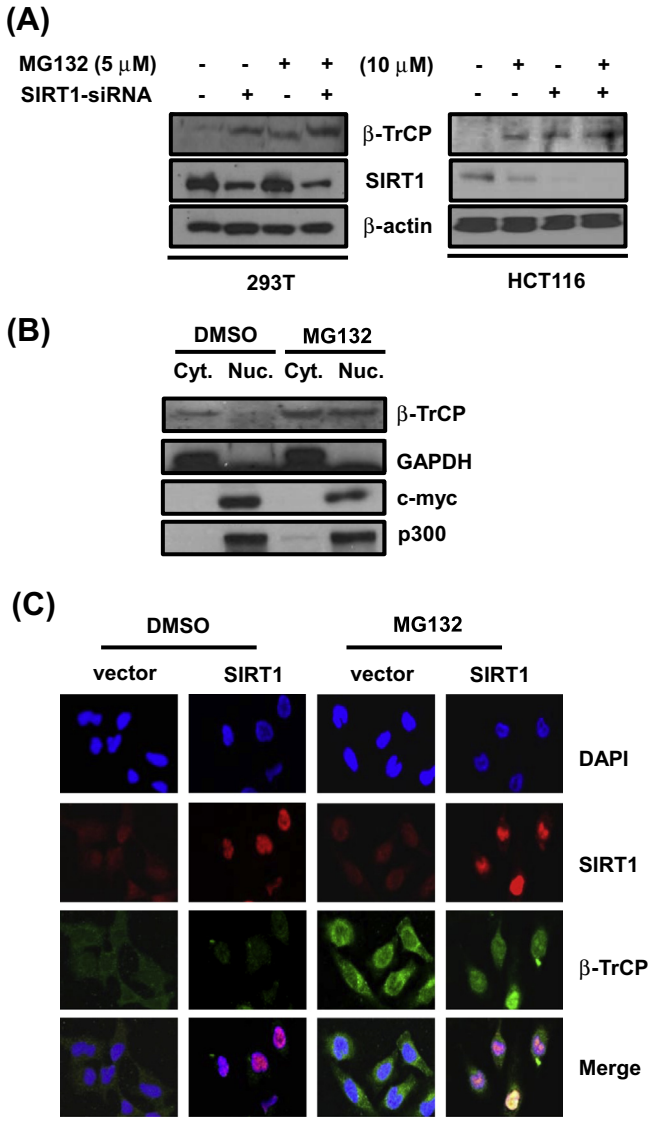


**Fig. 4.** SIRT1 suppression of β-TrCP accumulation is mediated by translational degradation. (A) Recovery of β-TrCP accumulation with MG132 treatment. β-TrCP recovery following treatment with MG132 was determined in 293T and HCT116 cells transfected with SIRT1- (+) and control-siRNAs (−). (B) Cellular localization of β-TrCP recovery. β-TrCP was detected both in the cytosol and nucleus of HeLa cells following treatment with MG132. GAPDH and c-myc/p300 were used as markers for fractionation of cytosol and nucleus, respectively. (C) Immunofluorescence analysis of β-TrCP localization. HeLa cells transfected with SIRT1 or empty vector were probed with either β-TrCP or SIRT1 antibody, and sequentially with fluorescent-tagged secondary antibodies. DAPI was used for counterstaining nuclei. The β-TrCP level and localization were determined in the absence (left two columns) and presence of MG132 (right two columns).

[5,6] and stress-activated kinase [7]. Under conditions where β-TrCP and hnRNP-U are associated, hnRNP-U stabilizes β-TrCP [33]. SIRT1-mediated suppression of β-TrCP observed in our experiments was attributed to the regulation of post-translational degradation of β-TrCP protein. Specifically, SIRT1 suppressed β-TrCP expression via promoting protein degradation. Notably, while β-TrCP protein was increased following SIRT1 depletion, the mRNA level was not altered. In addition, the suppressed β-TrCP level upon SIRT1 overexpression was recovered by proteasomal inhibition of protein degradation. These findings collectively present a novel mechanism of SIRT1-mediated regulation of β-TrCP via promotion of protein degradation.

## Acknowledgments

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.10.146.

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