약학박사 학위논문

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SIRT1 과의 직접 결합을 통한 Curcumin 의 인체 대장암 세포증식 억제 효능

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by

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ABSTRACT

Curcumin inhibits colon cancer progression through direct interaction with SIRT1

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Colorectal cancer (CRC) is the third most common cancer diagnosed and the third leading cause of cancer-related death in both males and females in the United States. It has been widely believed that frequent consumption of Western diet containing red meat and saturated fats or alcohol, lack of physical activity and obesity are the risk factors for CRC. Thus, the incidence rates of CRC have been gradually increased in recent years in the South Korea, which is partly attributable to Westernization of dietary habits. Even though the colonoscopy

for detection of colorectal polyps has been commonly used as a diagnostic method for CRC, recent studies have paid attention to usability of tumor-specific biomarkers as diagnostic and prognostic factors for CRC.

The SIRT protein family is an NAD^+-dependent class III HDAC which can deacetylate non-histone proteins as well as histones and consists of seven isoforms (SIRT1-7). Among these isoforms, SIRT1 was first reported to extend the life span of budding yeast in 1999. Subsequent studies demonstrated diverse physiological actions of SIRT1, such as metabolic regulation, differentiation and stress response. However, the role of SIRT1 in tumorigenesis is still controversial. Recent studies have revealed that SIRT1 is abnormally overexpressed in CRC tissues. Notably, the elevated levels of SIRT1 are significantly associated with not only tumor invasion and lymph node metastasis, but higher grades of malignancy and mutations of *KRAS* and *BRAF*, suggesting the prognostic utility of SIRT1. In the current study, I also confirmed that 27 out of 35 samples (77.14%) showed upregulation of SIRT1 in human colon tumor tissues compared with that of corresponding adjacent normal colon tissues as measured by immunofluorescent analysis. When SIRT1 was silenced in human CRC cell lines (HCT-116, HCT-15, DLD-1 and SW480) by transfection with SIRT1-specific small interfering RNA (siRNA), cell viability, migration, growth and invasion decreased. A xenograft mouse model revealed that the tumor volume and the tumor weight decreased in SIRT1-silenced xenograft

tumor compared with those observed in control xenograft tumor. These results suggest the oncogenic function of SIRT1 in the progression of CRC.

The use of natural compounds derived from edible plants has been considered as an attractive strategy to control tumor-promoting proteins. Interestingly, according to data published by World Health Organization (WHO) in 2003, the incidence of CRC is 10 times lower in India than in United states. Epidemiologic studies suggest that dietary phytochemicals, such as curcumin abundant in Indian food, may be responsible for the low risk of intestinal cancer in India. Curcumin, a yellow pigment present in the rhizome of turmeric (*Curcuma longa* L., Zingiberaceae) used in preparing the curry powder, is one of the most extensively investigated phytochemicals in regard to chemopreventive and antitumorigenic potential. It modulates the expression or activity of multiple proteins involved in cellular signal transduction pathways. It has been reported that curcumin can exert preventive effects against pathogenesis of several disorders by activating SIRT1 in cells or tissues of noncancerous origin. However, the relationship between curcumin and SIRT1 in CRC cells remains largely unknown. Thus, the purpose of this study was to investigate the effect of curcumin on the expression of SIRT1 and underlying molecular mechanisms in the context of inhibition of CRC progression.

When HCT-116 cells were treated with curcumin, the protein expression of SIRT1 was reduced while its mRNA transcript level was not changed, suggesting that SIRT1 is posttranslationally regulated by curcumin. Moreover, treatment of cells with curcumin significantly shortened the half-life of SIRT1, suggesting that curcumin destabilizes SIRT1 by enhancing its degradation rather than inhibiting *de novo* synthesis. Notably, the inhibitory effect of curcumin on SIRT1 protein was blunted by a proteasome-specific inhibitor MG-132, indicating that proteasomes are required for curcumin-induced degradation of SIRT1. This breakdown of SIRT1 was mediated through its ubiquitination both at endogenous and exogenous levels as measured by immunoprecipitation assay. These findings suggest that curcumin stimulates ubiquitin-dependent proteasomal degradation of SIRT1, thereby decreasing the protein stability.

It has been reported that increased protein expression of SIRT1 in CRC and hepatocellular carcinoma is attributable to enhanced protein stability rather than elevated mRNA expression of SIRT1, suggesting that abnormally stabilized SIRT1 could contribute to tumorigenesis. Nonetheless, much less is known about regulation of SIRT1 protein stability. Curcumin has two electrophilic α,β-unsaturated carbonyl groups, and hence can act as a Michael reaction acceptor. Considering tetrahydrocurcumin, a non-electrophilic analogue of curcumin, fails to exert the inhibitory effect on SIRT1 protein, structural feature of curcumin, namely, the α,β-

unsaturated carbonyl group plays an important role in reducing the stability of SIRT1. There are several reports suggesting that curcumin can directly modify cysteine thiols of cellular proteins by Michael addition, thereby modulating their functions. Based on these reports, recombinant human SIRT1 was incubated with curcumin followed by nano-LC-ESI-MS/MS analysis to investigate the modifiable residues of SIRT1 by curcumin. The data revealed modification of a single cysteine residue at 67 on SIRT1 by curcumin. Indeed, replacement of cysteine 67 of SIRT1 with alanine abolished the binding of curcumin to SIRT1. Moreover, the protein stability and clonogenicity of a mutant SIRT1 in which cysteine 67 was substituted by alanine were unaffected by curcumin. Taken together, these observations suggest that curcumin facilitates the proteasomal degradation of oncogenic SIRT1 through covalent modification of SIRT1, presumably at the cysteine 67 residue.

It has been suggested that the protein levels of SIRT1 and phospho-SIRT1 are higher in colorectal cancer tissues than adjacent normal tissues. Phospho-SIRT1 is positively correlated with a cellular proliferation marker, Ki67, indicating distinctive role of phospho-SIRT1 independently of total SIRT1 in the tumorigenesis of colon. It has also been reported that c-Jun N-terminal kinases 2 (JNK2) phosphorylates SIRT1 at serine 27 residue, thereby enhancing the protein stability of SIRT1 in human colon cancer (HCT-116) cells. Based on these reports, I hypothesized a possible involvement of dephosphorylation of SIRT1 in its

destabilization induced by curcumin. When HCT-116 cells were exposed to curcumin, phosphorylation level of SIRT1 at the serine 27 residue was reduced, together with the decrease in total level of SIRT1. Given the fact that phosphorylation defective mutant SIRT1 (SIRT1-S27A) had shortened half-life of SIRT1, and phosphomimetic mutant SIRT1 (SIRT1-S27D) was resistant against curcumin compared to wild-type of SIRT1, decreased phosphorylation level of SIRT1 was not due to its reduced total level of SIRT1. Immunoblot analysis showed that SP600125, a selective inhibitor of JNK, reduced the levels of phospho- and total SIRT1, but not the mRNA level, suggesting that JNK posttranslationally regulates SIRT1. Indeed, inhibition of JNK by SP600125 provoked ubiquitination-dependent degradation of SIRT1, resulting in its decreased stability. In parallel with these findings, anisomycin-induced activation of JNK prevented curcumin-mediated suppression of migration and growth of HCT-116 cells as well as SIRT1 by attenuating its ubiquitination. These data suggest that phosphorylation of serine 27 residue protects SIRT1 from curcumin-induced breakdown, which contribute to the maintenance of oncogenicity of HCT-116 cells against curcumin.

Taken together, curcumin can covalently modify SIRT1, preferentially at the cysteine 67 residue. Likewise, it can induce dephosphorylation of SIRT1 at the serine 27 residue through inhibition of JNK. These direct and indirect modifications of SIRT1 by curcumin can

collaboratively lead to proteasomal degradation of oncogenic SIRT1, which accounts for anti-cancer effects of curcumin in terms of suppression of cell migration and growth.

Key words

Colorectal cancer; SIRT1; Curcumin; Thiol modification; c-Jun N-terminal kinase; Phosphorylation

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List of Abbreviations

4-Hydroxy-2-nonenal, 4-HNE

Aberrant crypt foci, ACF

Active regulator of SIRT1, AROS

AMP-activated protein kinase, AMPK

Ca^{2+}/calmodulin-dependent protein kinase kinase, CaMKKβ

Carbamoyl phosphate synthetase 1, CPS1

Casein kinase II, CK2

Colorectal cancer, CRC

Constitutive photomorphogenic 1, COP1

Cyclic adenosine monophosphate, cAMP

Cyclin-dependent kinase, CDK

Cycloheximide, CHX

Deleted in breast cancer-1, DBC1

Dithiothreitol, DTT

Dual-specificity tyrosine phosphorylation-regulated kinases, DYRKs

Epigallocatechin gallate, EGCG

Extracellular signal regulated kinase, ERK

Fetal bovine serum, FBS

Forkhead box protein O1, FoxO1

Forkhead box protein O3, FoxO3

Glucokinase regulatory protein, GKRP

Glutamate dehydrogenase, GDH

Glutaredoxin-1, GRX1

Glutathione, GSH

Glyceraldehyde 3 phosphate dehydrogenase, GAPDH

Hematoxylin and eosin, H&E

Histone deacetylase, HDAC

Interleukin, IL

c-Jun N-terminal kinase, JNK

Long-chain acyl CoA dehydrogenase, LCAD

Malonyl CoA decarboxylase, MCD

Multidrug resistance 1, MDR1

N-acetyl-l-cysteine, NAC

Nicotinamide adenine dinucleotide, NAD

Nitric oxide, NO

Nitrosoglutathione, GSNO

Nuclear factor of activated T-cells, NFAT

Peroxisome proliferator-activated receptor gamma coactivator 1 α, PGC-1α

Phosphate-buffered saline, PBS

Proliferating cell nuclear antigen, PCNA

Propidium iodide, PI

Protein inhibitor of activated STAT 3, PIAS3

Protein kinase A, PKA

Redox factor-1, Ref-1

Retinoblastoma protein, Rb

Reverse transcription-polymerase chain reaction, RT-PCR

Signal transducer and activator of transcription 3, STAT3

Silent mating type information regulation 2, *S. Cerevisiae*, homolog, sirtuin (also known as SIRT)

Small interfering RNA, siRNA

Small ubiquitin-related modifier, SUMO

Sodium dodecyl sulfate polyacrylamide gel electrophoresis, SDS-PAGE

Chapter I

Posttranslational modification of SIRT1 and its modulation by chemopreventive and anti-carcinogenic natural products

1. Introduction

The SIRT proteins are present in all kingdoms of life and are broadly conserved from yeast to humans. They consist of at least seven isoforms (SIRT1-7) and possess either NAD^+-dependent histone/non-histone deacetylase activity or ADP-ribosyltransferase activity. Structurally, they contain a highly conserved catalytic core domain and variable N- and C-terminal domains as illustrated in **Fig. 1-1**. **Table 1-1.** summarizes differences in their subcellular localization, enzymatic activity and functions. SIRT1 has a multifaceted role in physiologic and pathologic processes, such as cellular senescence (1), apoptosis (2), inflammation (3) and energy metabolism (4). SIRT1 contains two nuclear localization signal sequences and two nuclear export signal sequences, so it freely resides in both nuclear and cytosolic compartments in the cells. Interestingly, it has been reported that abnormally overexpressed SIRT1 in cytoplasm could promote the malignancy of several cancers including those derived from colon (5), prostate (6) and ovarian cancer (7). Likewise, SIRT2 can shuttle between nucleus and cytoplasm to regulate the cardiac homeostasis (8), glucose uptake (9) and differentiation (10). SIRT3, 4 and 5 are predominantly localized in mitochondrial matrix and involved mainly in the regulation of cellular metabolism such as lipid anabolism (11) and urea cycle (12). SIRT6 and 7 are the nuclear proteins that are known to play a role in telomere maintenance (13) and pre-rRNA processing (14).

Of the aforementioned SIRT isoforms, SIRT1 has been most extensively investigated, and there is a growing number of its substrates and target molecules. It exerts influence on the protein acetylation dynamics by deacetylating the substrate proteins, thereby carrying out the multiple roles in many physiological and pathophysiological conditions. Considering its multifarious effects on various cellular events, expression or activity of SIRT1 needs to be properly and precisely regulated. Because SIRT1 is classified as an NAD^+-dependent deacetylase, NAD^+ is primarily regarded as a factor that controls the activity of SIRT1. In addition, independent of biosynthesis, concentrations and availability of intracellular NAD^+, endogenous proteins can also modulate the activity of SIRT1. One such molecule is active regulator of SIRT1 (AROS) that binds to the allosteric site located in the N-terminus of SIRT1, whereby increasing its activity (15, 16). AROS-SIRT1 complex formation provokes conformational changes in SIRT1. This allows SIRT1 to attain the structure most favourable for its catalytic activity without obstructing the interaction between SIRT1 and its substrates (15). On the other hand, deleted in breast cancer-1 (DBC1) directly interacts with the catalytic domain of SIRT1, resulting in inhibition of SIRT1 activity (17). In addition to these endogenous regulators of SIRT1 activity, posttranslational modifications are recognized as an important means to control the function of SIRT1. Since SUMOylation of SIRT1 was first reported as a posttranslational modification of SIRT1 to regulate its deacetylase activity (18),

many other chemical alterations have been suggested so far. Posttranslational modifications that occur in various residues of proteins can alter the nature of the protein in diverse aspects such as activity, stability, subcellular localization and binding affinity for substrates. Analysis of affinity-purified FLAG-SIRT1 by use of mass spectrometry suggests that SIRT1 could be phosphorylated at multiple residues by various kinases. Currently, eighteen residues of serine/threonine have been reported to be the targets for SIRT1 phosphorylation (19-23). Furthermore, SIRT1 contains nineteen cysteine residues, and some of which are susceptible to redox modification. Due to the high reactivity of the cysteine thiol toward reactive oxygen species (ROS) and electrophiles, SIRT1 is prone to be structurally modified through oxidation, *S*-nitrosation, *S*-glutathiolation and *S*-sulfenation. Like phosphorylation, modifications occurring at cysteine residues are closely implicated in not only modulation of intracellular signaling, but the balance of cellular redox environment.

In this chapter, I highlighted posttranslational modifications of specific residues of SIRT1 by intracellular molecules, particularly at serine/threonine and cysteine, in diverse biological contexts. Additionally, I described effects of some natural phytochemicals on SIRT1 in *in vitro* and *in vivo* experimental models of aging, inflammation and cancer.

2. Posttranslational modifications of SIRT1 by intracellular molecules

2.1. Posttranslational modifications of serine/threonine residues

2.1.1. c-Jun N-terminal kinases (JNKs)

Ford *et al*. reported that JNK2 is associated with phosphorylation of SIRT1 at the serine 27 residue, which contributed to enhanced protein stability of SIRT1 in human colon cancer (HCT-116) cells (24). According to this study, not only serine 27 but serine 47 was identified by mass spectrometry as a putative phosphorylation site, but phosphorylation of SIRT1 at serine 47 showed no correlation with its protein stability. Interestingly, JNK1, another isoform of the JNK family, phosphorylates mouse SIRT1 at serine 46 (corresponds to serine 47 of human SIRT1), thereby inducing ubiquitination-dependent degradation of SIRT1 in mouse adipocytes (3T3-L1) and human embryonic kidney (HEK293) cells (25). The half-life of SIRT1 protein was prolonged in JNK1-knockout (KO) murine embryonic fibroblasts (MEFs), further corroborating the involvement of JNK1 in SIRT1 phosphorylation (25).

2.1.2. Ca^{2+}/calmodulin-dependent protein kinase kinase (CaMKK)β

Both serine 27 and 47 of SIRT1 can also be phosphorylated by CaMKKβ under pulsatile shear stress (PS) mimicking atheroprotective flow to exert antioxidative effect in human umbilical vein endothelial cells (HUVECs) (26). PS-induced phosphorylation of SIRT1 at serine 27 and 47 is accompanied by its increased protein level without change in its mRNA level.

Subsequent *in vitro* kinase assay and nano-LC-MS/MS analysis revealed that CaMKKβ could directly phosphorylate SIRT1 at aforementioned residues. Thus, the increased SIRT1 protein stability in response to PS was abrogated in CaMKKβ$^{-/-}$ MEFs. In parallel with this finding, the expression of anti-oxidative genes such as *superoxide dismutase*, *catalase*, *Nrf2*, *heme oxygenase-1* and *thioredoxin 1* were upregulated in SIRT1$^{-/-}$ MEFs expressing phosphomimetic mutant SIRT1 (S27D/S47D) compared with that of control (26). Taken these findings all together, CaMKKβ dependent phosphorylation of SIRT1 is likely to contribute to antiatherogenic property in vascular endothelial cells.

2.1.3. AMP-activated protein kinase (AMPK)

It has been shown that AMPK directly phosphorylates SIRT1 at threonine 344 residue, leading to its release from the endogenous inhibitor DBC1 in human osteosarcoma (U2OS) cells, thereby inducing the deacetylation-dependent inactivation of p53 (27). Although DBC1 can also be a substrate of AMPK, the fate of SIRT1-DBC1 complex depends on the phosphorylation status of SIRT1 at the threonine 344. Contrary to these phenomena, Lee *et al*. reported that SIRT1 is phosphorylated by AMPK at the same residue and subsequently inactivated in human hepatocellular carcinoma (HepG2) cells (28). In this study, a phosphomimetic mutant of SIRT1 in which threonine 344 is substituted by glutamic acid

(T344E) failed to exert an inhibitory effect on the transcriptional activity of p53 compared with that of a wild type or non-phosphorylatable mutant of SIRT1 (T344A) in the p53 luciferase reporter assay. Thus, phosphorylation of SIRT1 at threonine 344 is considered to decrease its deacetylase activity, resulting in enhancement of p53 acetylation as well as apoptosis in HepG2 cells.

2.1.4. Protein kinase A (PKA)

SIRT1 is known to take part in fatty acid oxidation to protect cells from metabolic stress. In this process, the catalytic activity of SIRT1 toward peroxisome proliferator-activated receptor gamma coactivator 1 α (PGC-1α) is positively regulated by the cyclic adenosine monophosphate (cAMP)-dependent PKA (19). Mass spectral analysis of immunoprecipitated SIRT1 protein revealed that the only serine 434 residue was exclusively phosphorylated as a consequence of an adenylyl cyclase activator (forskolin)-induced increase of cellular cAMP in U2OS cells. In accordance with this result, SIRT1-KO MEF cells harbouring mutation of serine 434 to alanine failed to induce deacetylation/activation of PGC-1α and subsequent transcription of lipid metabolism genes, supporting the requirement of SIRT1 phosphorylation at the serine 434 for the lipid oxidation process.

2.1.5. Dual-specificity tyrosine phosphorylation-regulated kinases (DYRKs)

Two anti-apoptotic DYRK members, DYRK1A and DYRK3, activate the SIRT1 through phosphorylation at threonine 522 to promote the cell survival (20). Thus, pro-survival activity of SIRT1 is associated with its increased phosphorylation, especially at the threonine 522 residue. Dephosphorylation of SIRT1 in cells in which the threonine 522 is replaced by valine prevented DYRK/SIRT1-induced deacetylation of p53 and cell survival. Additionally, hypophosphorylation of SIRT1 by knockdown of DYRK1A and DYRK3 using specific small interfering RNA (siRNA) sensitized human osteosarcoma (U2OS) cells toward etoposide-induced cell death (20). Later, it has been suggested that phosphorylation of the threonine 522 residue of SIRT1 is closely related to its conformational stability as well as aforementioned binding affinity for p53 (29). To elucidate the relevance of phosphorylation as a regulatory mechanism in controlling the catalytic activity of SIRT1, the authors of the above study utilized purified three recombinant proteins, i.e., wild-type SIRT1 (SIRT1-WT), a non-phosphorylation mimetic mutant SIRT1 (SIRT1-T522A) and a phosphorylation mimetic mutant SIRT1 (SIRT1-T522E). In an *in vitro* enzymatic assay, SIRT1-T522E exerted higher ability to remove the acetyl group from acetylated p53 fusion proteins than did SIRT1-WT or SIRT1-T522A. Enhanced deacetylase activity of SIRT1-T522E appeared to be achieved by maintaining the monomeric state of the SIRT1 protein to block the formation of aggregates.

2.1.6. CyclinB/Cyclin-dependent kinase 1 (CDK1)

Sasaki and colleagues reported that dephosphorylation of SIRT1 by phosphatases, such as calf intestinal phosphatase or lambda phosphatase, led to its decreased deacetylase activity (30). Comparative analysis of SIRT1 amino acid sequences revealed that threonine 530 and serine 540 are relatively well conserved among the orthologs of SIRT1. Interestingly, these two residues fit the consensus sequence for a CDK substrate (S/T*-P or S/T*-P-x-K/R). These investigators verified complex formation between endogenous or exogenous SIRT1 and cyclin/CDK, specifically cyclinB/CDK1 by co-immunoprecipitation assay (30). Even though they clearly showed cyclinB/CDK1-mediated phosphorylation of SIRT1 at the threonine 530 and serine 540 residues, no significant differences were found to exist between wild-type SIRT1 (SIRT1-WT) and the threonine 530 to alanine and serine 540 to alanine mutant (SIRT1-T530A/S540A) as assessed by the NAD^+-dependent *in vitro* deacetylase activity assay. The proliferative capacity which was defected in SIRT1-deficient cells (SIRT1-KO MEFs or ES cells) was restored by transfection of cells with wild-type SIRT1, but not with the SIRT1-T530A/S540A, suggesting that phosphorylation of SIRT1 is associated with mitotic activity of the cell. This incongruity between *in vitro* and *in vivo* studies might be that these residues could indirectly influence the catalytic activity of SIRT1. The authors suggested that phosphorylation of the threonine 530 and serine 540 modulates the activity of SIRT1 by

altering the accessibility of substrate molecules to the catalytic groove of this deacetylase not by affecting the SIRT1 itself.

2.1.7. Casein kinase II (CK2)

Casein kinase II (CK2) also participates in phosphorylation of SIRT1 mainly at the serine 659 and 661 residues (31). Recombinant CK2 phosphorylated immunoprecipitated FLAG-tagged SIRT1 in human cervical cancer (HeLa) cells as verified by an *in vitro* kinase assay. Moreover, knockdown of CK2 using siRNA or pharmacologic inhibition reduced the phosphorylation intensity of immunoprecipitated endogenous SIRT1 from HeLa cells labeled with [□-^{32}P] ortho-phosphate (31). Subsequent amino acid sequence analysis indicated serine 659, 661 and 684, which are located within the CK2 consensus sequence (S/TxxD/E), as putative CK2 phosphorylation sites. Single mutation of these residues revealed that S659A and S661A mutants showed reduced radioactive phosphorylation signal compared with wild type SIRT1, whereas the S684A mutant did not exert such an effect on phosphorylation intensity *in vitro*. These findings suggest that SIRT1 undergoes phosphorylation predominantly at both serine 659 and 661 by CK2. The physiological relevance of CK2-mediated phosphorylation of SIRT1 was later elucidated by Kang and colleagues (32). Phosphorylation of SIRT1 conferred

resistance to etoposide-induced apoptosis in human non-small cell lung cancer (NSCLC) (H1299) cells, and this effect was mediated through significant deacetylation of p53.

2.2. Posttranslational modifications of cysteine residues

2.2.1. S-Nitrosoglutathione (GSNO) and reduced glutathione (GSH)

GSNO, formed by reaction of nitric oxide (NO) and GSH, affected resveratrol-induced activation of SIRT1 by directly modifying the cysteine 67 residue *in vitro* (33). Even though GSNO-induced oxidation of cysteine 67 did not affect the basal activity of SIRT1, it dampened the responsiveness of SIRT1 to resveratrol, resulting in decreased activity of SIRT1. Meanwhile, cysteine 61 of mouse SIRT1, which corresponds to cysteine 67 of human SIRT1, is prone to be modified by GSH, together with cysteine 318 and 613 (correspond to cysteine 326 and 623 of human SIRT1, respectively) (34). Reduction of GSH-SIRT1 adduct by glutaredoxin-1 (GRX1) overexpression preserved activity of SIRT1 in HepG2 cells. In addition, high-fat and high-sucrose diet-fed C57BL/6J mice showed increased accumulation of GSH-protein adducts with concomitant reduction in the SIRT1 activity in the fatty liver of mice, suggesting oxidative inactivation of SIRT1 under metabolically stressed conditions.

2.2.2. Redox factor-1 (Ref-1) and GSNO

Redox factor-1 (Ref-1), as a cellular reductant, stimulated SIRT1 activity by maintaining SIRT1 in the reduced form (35). The catalytic domain of SIRT1 has a Zn^{2+}-tetra-thiolate motif containing cysteine 371, 374, 395 and 398 residues, which is highly conserved from yeast to human. When cysteine 371 and 374 residues in this tetrathiolate motif were mutated to serine, Ref-1 could not prevent the loss of SIRT1 activity caused by hydrogen peroxide-induced oxidative stress in HUVEC cells, suggesting that sustained maintenance of SIRT1 in a reduced form is important for retaining its optimal catalytic activity under oxidative stress. However, *S*-nitrosation of SIRT1 at cysteine 395 and 398 residues that are other constituents of $Zn2^{+}$-tetra-thiolate, correlated with the inhibition of its catalytic activity (36). These modifications, which result from the attachment of NO to cysteine residues of SIRT1, provoked conformational changes of SIRT1. As a result, Zn^{2+} was released from Zn^{2+}-tetra-thiolate of SIRT1, and this hampered the binding of a substrate molecule and NAD^{+}, lowering the activity of SIRT1.

2.2.3. SNO–Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

It has been suggested that GAPDH can donate NO to SIRT1, thereby affecting the activity of SIRT1 (37). GAPDH was first nitrosylated by NO and then the resulting SNO–GAPDH complex physiologically transnitrosylated SIRT1 at the cysteine 387 and 390. This led to the

inhibition of SIRT1 enzymatic activity and concomitantly transcriptional activity of PGC-1α, a well-known substrate of SIRT1 (37).

2.2.4. 4-Hydroxy-2-nonenal (4-HNE)

It has been suggested that SIRT1 was inactivated prior to protein degradation under certain conditions, such as cigarette smoke (CS)-mediated oxidative stress. Pretreatment of human bronchial epithelial (BEAS-2B) cells with a thiol reducing agent *N*-acetyl-l-cysteine (NAC) prevented cigarette smoke extract (CSE)-induced loss of SIRT1, suggesting involvement of oxidative modifications in CSE-mediated degradation of SIRT1 (38). Clinical observations of smokers and COPD patients showed that 4-HNE, which is a CSE-induced lipid peroxidation product, formed covalent adducts with SIRT1 in lungs (39). Thus, MALDI TOF/TOF mass spectrometry identified SIRT1 carbonylated by 4-HNE, particularly at the cysteine 482 residue.

3. SIRT1 as molecular target for health beneficial effects of natural products

3.1. Aging

The link between SIRT1 and longevity was first discovered in budding yeast in 1999 (40). Subsequent studies using laboratory rodents demonstrated the essential role of SIRT1 in

prolonged life span under caloric restriction condition (41-43). Resveratrol found in wine has been reported to have health benefits as known from 'French paradox'. Since identification as one of the most prominent SIRT1-activating compounds based on screening of small molecule libraries by Howitz and colleagues in 2003 (44), resveratrol has received tremendous attention regarding lifespan-extending effect and its mechanism. Resveratrol treatment potentiated mitochondrial function and mitochondrial citric acid cycle enzyme activity (e.g., succinate dehydrogenase, citrate synthase, etc.) which account for its capability to extend the life span of high-fat diet-fed C57Bl/6J mice (45). These effects were dependent on resveratrol-induced deacetylation of PGC-1α to a large extent, thus authors investigated the involvement of SIRT1, which is responsible for the deacetylation of PGC-1α. Indeed, effects of resveratrol were lost in SIRT1-KO MEF cells and mouse myoblast (C2C12) cells transfected with a specific short hairpin RNA against SIRT1. In another study, resveratrol lowered the risk of death from the high-calorie diet-fed C57BL/6NIA mice through enhancement of SIRT1 activity (46). Epigallocatechin gallate (EGCG), a major antioxidant ingredient found in green tea, has been known to possess anti-aging activity. Niu *et al*. have reported that EGCG treatment allowed healthy rats to live longer by 12.5 weeks compared with vehicle treatment (47). This beneficial effect was attributed to the amelioration of age-associated oxidative stress and tissue damage in the liver and kidney by decreasing the levels

of tumor necrosis factor-α (TNF-α), interleukin (IL)-6, ROS and malondialdehyde, a lipid peroxidation product. Certainly, SIRT1 was upregulated the liver and kidney tissues of rats treated with EGCG, suggesting possible involvement of SIRT1 activation in health benefits of EGCG. Antcin M present in the edible mushroom (*A. salmonea*). Antcin M treatment not only attenuated high-glucose-induced SIRT1 degradation and cellular senescence in human normal dermal (CCD966SK) fibroblasts, but also extended the life span in *C. elegans* (48). In other model organisms, such as fruit fly (*D. melanogaster*), alkylresorcinols, the natural phenolic lipids found in whole grains, delayed the aging process by stimulating catalytic activity of SIRT1 (49). The ability of alkylresorcinols to extend the life span was abolished in flies with *Sir2*-deficient genotype.

3.2. Inflammation

NF-κB has been considered as a master regulator in controlling the inflammatory responses due to its critical role in production of diverse proinflammatory cytokines including IL-1β, IL-6 and TNF-α. It has been reported that SIRT1 contributed to resolution of NF-κB-dependent inflammation through deacetylation of the RelA/p65 subunit of NF-κB at the lysine 310 residue, thereby inhibiting its transcriptional activity (3, 50). Thus, it is plausible that

SIRT1 mediates the anti-inflammatory effects of some natural products by hindering the activation of NF-κB.

Hinokitiol is a tropolone-related natural compound isolated from the wood of *Chamaecyparis taiwanensis* and has been shown to possess antioxidant and anti-inflammatory activities toward various toxic stimuli. Lipopolysaccharides (LPS) treatment led to translocation of NF-κB into the nucleus and subsequent induction of pro-inflammatory genes, such as IL-6 and TNF-α, in normal human epidermal keratinocyte (NHEK) cells (51). These effects were decreased by hinokitiol treatment. However, the hinokitiol-induced blockade of the NF-κB pathway was blunted by pharmacological inhibition or siRNA-mediated knockdown of SIRT1, suggesting that the anti-inflammatory effect of hinokitiol on LPS-stimulated inflammatory responses is mediated through activation of SIRT1.

Dong and colleagues have suggested requirement of SIRT1 in alleviation of high-fat diet-induced adipose tissue inflammation by quercetin, a bioflavonoid abundant in fruits and vegetables (52). High-fat diet-induced degradation of SIRT1 was attenuated by quercetin in epididymis adipose tissues of mice, thereby contributing to antagonization of M1 polarization and inflammation of macrophages (52).

3.3. Cancer

Even though SIRT1 has been known to exert beneficial roles in non-malignant conditions, the role of SIRT1 in cancer development and progression is still under debate. SIRT1 acts as a tumor suppressor (53-55), but is also known as an oncogene (56-58). It has been shown that SIRT1 exerts tumor suppressive functions by deacetylating and suppressing the transcriptional activity of NF-κB and signal transducer and activator of transcription 3 (STAT3) (59-62). On the other hand, the levels of SIRT1 are elevated in many types of cancer, and it negatively regulates p53 and retinoblastoma protein (Rb), well-known tumor suppressors, via deacetylation-dependent inactivation (2, 63, 64). Accordingly, there are conflicting observations of the effects of naturally occurring bioactive compounds on SIRT1 activity/expression in various malignant cells.

Curcumin (diferuloylmethane), a yellow-colored polyphenol derived from the spice turmeric (*Curcuma longa* L., Zingiberaceae) induced activation of not only SIRT1, but ATM/CHK2-mediated apoptotic signaling in head and neck squamous cell carcinomas (HNSCC) (FaDu and Cal27) cells (65). Curcumin apparently inhibited growth and proliferation of HNSCC cells as well as growth of FaDu tumor xenografts. However, the role of SIRT1 in ATM/CHK2 signaling remains to be further elucidated. The authors did not investigate relevance of curcumin-induced activation of SIRT1 in the ATM/CHK2-mediated apoptosis triggered by curcumin. It has been known that ATM/CHK2 signaling is activated

following DNA damage or genotoxic stress and determines the cell fate among the cell cycle arrest, apoptosis and DNA repair. Not only ATM could activate SIRT1 through phosphorylation of transcription factor E2F1 (66) but also SIRT1 could contribute to the ATM activation (67). In both cases, SIRT1 enhanced DNA repair activity, thus its activation evoked cell survival rather than apoptosis. In another study, curcumin facilitated the proteasomal degradation of SIRT1 in human colon cancer (HCT-116) cells. Curcumin can act as a Michael reaction acceptor due to presence of electrophilic α,β-unsaturated carbonyl groups. Thus, nucleophilic cysteine 67 residue of SIRT1 was directly modified by curcumin. This structural modification resulted in decreased protein stability of SIRT1, together with inhibition of migration and growth of HCT-116 cells (68).

Resveratrol exerted a chemopreventive effect via activation of SIRT1 in human colon cancer (HCT-116 and SW480) cells (69). Resveratrol showed the inhibitory effects on proliferation, migration and invasion of HCT-116 and SW480 cells by suppressing the NF-κB signaling, which was abolished by knockdown of SIRT1. Similarly, resveratrol decreased the viability and induced apoptosis in human chondrosarcoma (JJ012) cells by upregulating the protein expression of SIRT1 and subsequently inhibiting the RelA/p65 subunit of NF-κB (70). In another study, resveratrol significantly elevated the level of miR-34a, a tumor suppressive microRNA, resulting in suppression of protein synthesis of transcription factor E2F3 via RNA

interference in human colon cancer (DLD-1) cells (71). As SIRT1 is a target gene of E2F3, the expression of SIRT1 was consequently downregulated by resveratrol treatment, leading to inhibition of growth of DLD-1 cells. Additionally, SIRT1 has been shown to play a role in chemoresistance of some cancer cells. Overexpression of SIRT1 increased the nuclear level of the transcription factor, forkhead box protein O1 to induce upregulation of multidrug resistance 1 (MDR1) (72).

Amurensin G, a natural compound derived from wild grape (*Vitis amurensis)* inhibited the activity of SIRT1 in doxorubicin-resistant breast cancer (MCF-7/ADR) cells. As a consequence, forkhead box protein O1-dependent expression of MDR1 was suppressed by Amurensin G treatment, and these effects collaboratively led to inhibition of the xenograft tumor growth in the nude mice bearing MCF-7/ADR cells by restoring the responsiveness to doxorubicin (72) .

4. Concluding remarks

SIRT1 appears to play beneficial roles in normal physiological or moderate inflammatory conditions. However, the role of SIRT1 in malignant transformation is still uncertain. Many literatures suggest both protective and detrimental roles of SIRT1 in malignant tumors. Recent studies have demonstrated that SIRT1 is abnormally overexpressed in various types of human

malignancies, including breast (73), prostate (74), liver (75) and colon cancer (76), and high levels of SIRT1 are associated with the lymph node metastasis and poor prognosis (76-78). It has also been reported that phospho-SIRT1 is highly detected in colorectal cancer tissues compared to normal tissues and positively associated with Ki-67, a proliferation index, suggesting clinicopathologic relevance of posttranslational modification of SIRT1 (79). Posttranslational modifications, including phosphorylation, oxidation/reduction, carbonylation, nitrosylation, etc. have been well described as a regulatory modification due to their impact on activity, stability and subcellular localization of SIRT1 and also its binding affinity for substrate proteins. Thus, it is presumable that distinctive posttranslational modifications of SIRT1 by different distribution of intracellular molecules in normal and cancer cells, could contribute to the cancer-specific function of SIRT1.

The use of natural compounds derived from edible plants as a supplement to chemotherapy has been considered as an attractive strategy to improve effectiveness of chemotherapeutics while minimizing their side effects. However, the molecular mechanisms underlying regulation of SIRT1 in malignant processes by natural products remain to be further elucidated. Accumulating evidence for effects of some natural products on oncogenic SIRT1 will provide better understanding of the potential of SIRT1 as a chemotherapeutic target.

Table 1-1. Differences of the SIRT family in their subcellular localization, enzymatic activity and functions

Sirtuin	Subcellular localization	Enzymatic activity	Targets	Functions	Ref.
SIRT1	Nuclear/ cytoplasmic	Deacetylase	p53	Cellular senescence	(1)
				Apoptosis	(2)
			p65	Inflammation	(3)
			PGC-1α	Energy metabolism	(4)
SIRT2	Nuclear/ cytoplasmic	Deacetylase	NFAT	Cardiac homeostasis	(8)
			GKRP	Glucose uptake	(9)
			Slug	Basal differentiation	(10)
SIRT3	Mitochondrial	Deacetylase	LCAD	Fatty acid oxidation	(69)
			GSK-3β	Mitochondria-mediated apoptosis	(70)
SIRT4	Mitochondrial	ADP-ribosyltransferase	MCD	Lipid homeostasis	(11)
			GDH	Insulin secretion	(71)
SIRT5	Mitochondrial	Deacetylase	CPS1	Urea Cycle	(12)
			FoxO3	Apoptosis	(72)
SIRT6	Nuclear	Deacetylase	FoxO1	Gluconeogenesis	(73)
		ADP-ribosyltransferase	H3K9	Telomere maintenance	(13)
SIRT7	Nuclear	Deacetylase	H3K18	Oncogenic transformation	(74)
			U3-55k	Pre-rRNA processing	(14)

Table 1-2. Regulation of SIRT1 function by posttranslational modifications

Type of modification	Residue	Associated molecules	Effect on function of SIRT1 protein	Ref.
Phosphorylation	Ser27	JNK2 CaMKKβ	Enhanced protein stability Enhanced protein stability	(24) (26)
Phosphorylation	Ser47	JNK1 CaMKKβ	Degradation Enhanced protein stability	(25) (26)
S-Glutathionylation	Cys67	GSNO	Did not affect the basal activity of SIRT1, it dampened the responsiveness of SIRT1 to resveratrol, resulting in decreased catalytic activity of SIRT1	(33)
S-Glutathionylation	Cys67 Cys326 Cys623	GSH	Decreased catalytic activity	(34)
Phosphorylation	Thr344	AMPK	Enhanced catalytic activity	(27)
Phosphorylation	Thr344	AMPK	Decreased catalytic activity	(28)
Reduction	Cys371 Cys374	Ref-1	Enhanced catalytic activity	(35)
S-Nitrosylation	Cys387 Cys390	SNO–GAPDH	Decreased catalytic activity	(37)
S-Nitrosation	Cys395 Cys398	GSNO	Decreased catalytic activity	(36)
Phosphorylation	Ser434	PKA	Enhanced catalytic activity	(19)
Carbonylation	Cys482	4-HNE	Inactivation and degradation	(38, 39)
Phosphorylation	Thr522	DYRK1A DYRK3	Enhanced catalytic activity	(20, 29)
Phosphorylation	Thr530 Ser540	CyclinB/CDK1	Enhanced catalytic activity	(30)
Phosphorylation	Ser659 Ser661	CK2	Enhanced catalytic activity	(31, 32)

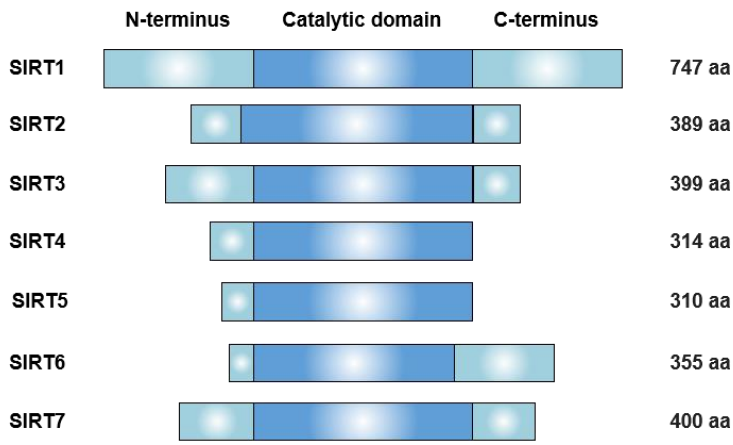

Fig. 1-1. The SIRT family. SIRT1 proteins contain a highly conserved catalytic core domain, variable N- and C-terminal domains.

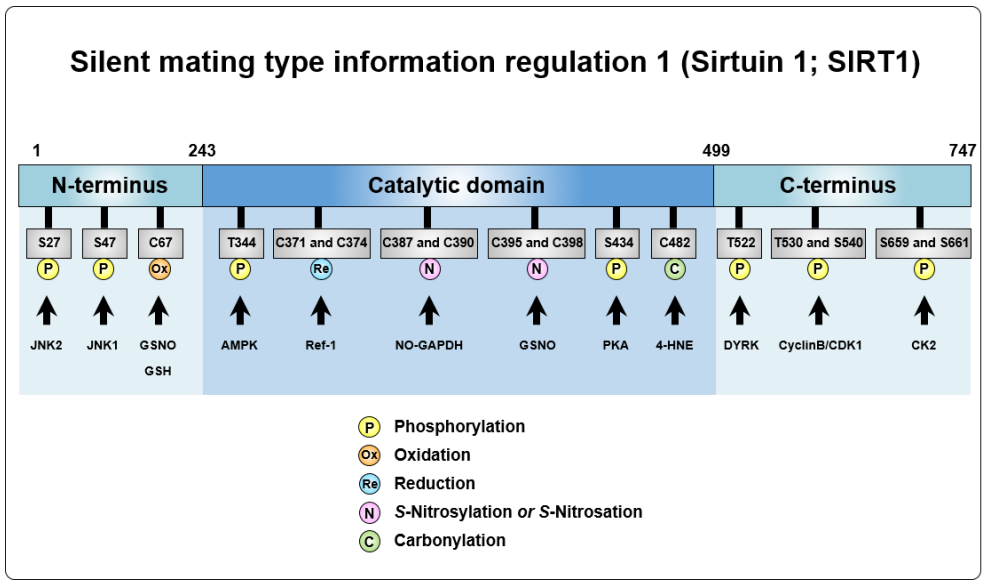

Fig. 1-2. Posttranslational modification of SIRT1 by intracellular signaling molecules. SIRT1 protein consists of 747 amino acids and is commonly divided into three functional domains, N-terminal domain, catalytic domain and C-terminal domain. SIRT1 can be modified by various posttranslational modifications, leading to alteration in enzymatic activity or expression of SIRT1. In general, cysteine residues are susceptible to oxidation/reduction, carbonylation, *S*-nitrosylation and *S*-nitrosation. Likewise, serine and threonine residues are particularly sensitive to phosphorylation.

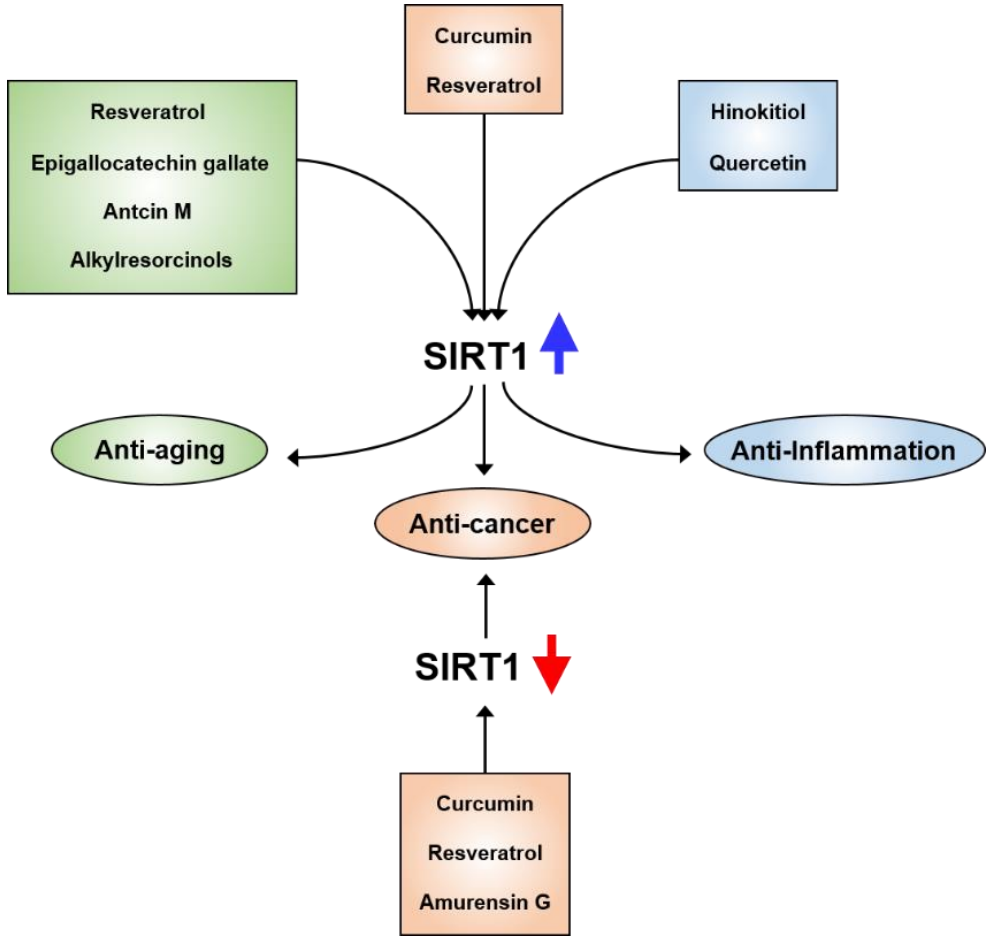

Fig. 1-3. Regulation of SIRT1 by various natural compounds in aging, inflammation and cancer.

5. References

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PURPOSE OF THE STUDY

SIRT1, an NAD^+-dependent histone/protein deacetylase, has diverse physiological actions. Recent studies have demonstrated that SIRT1 is abnormally overexpressed in colorectal cancer (CRC), and high levels of SIRT1 are associated with the lymph node metastasis and poor prognosis, suggesting its oncogenic potential. However, the molecular mechanisms by which overexpressed SIRT1 induces the progression of CRC and its inhibition remain largely unknown. In the present study, I thus elucidated the role of SIRT1 in colon cancer progression. Furthermore, I investigated the effect of curcumin, a naturally occurring bioactive compound found in turmeric, on the expression of SIRT1 and underlying molecular mechanisms in the context of inhibition of migration and growth of human colon cancer cells. In particular, the present study aims to elucidate the importance of structural feature of curcumin in the regulation of SIRT1 as well as oncogenicity of human colon cancer cells.

Chapter II

Oncogenic function of SIRT1 in colon cancer: a possible involvement of transcription factor STAT3

1. Abstract

SIRT1 is an NAD^+-dependent histone/protein deacetylase that has multiple physiological actions, such as inflammation, energy metabolism, lipid peroxidation, apoptosis, differentiation and stress response. This pleiotropic effects of SIRT1 may be attributed to a broad range of protein substrates residing in nucleus and cytoplasm. The role of SIRT1 in carcinogenesis, however, is still controversial. Recent studies have demonstrated that SIRT1 is highly expressed in several malignancies, suggesting that it may act as a tumor promoter. Here I showed that SIRT1 is overexpressed in colorectal cancer tissues compared with normal colon tissues and acts as an important regulator of colon cancer cell growth and proliferation. SIRT1 depletion decreased cell viability, migration, neoplastic growth and invasion of colon cancer cells. Additionally, silencing of SIRT1 resulted in decreased production of pro-inflammatory cytokines which are involved in growth and proliferation of colon cancer cells, particularly, interleukin (IL)-1β, 6 and 8. It has been reported that signal transducer and activator of transcription 3 (STAT3) is a major transcription factor that controls the expression of IL-6 and 8 in various cancer cells. This prompted me to consider the possibility that STAT3 is regulated by SIRT1. When HCT-116 cells were transiently transfected with SIRT1 siRNA, the protein expression of STAT3 was reduced while its mRNA transcript level remained unchanged, suggesting posttranslational regulation of STAT3 by SIRT1. In addition,

implantation of SIRT1-silenced HCT-116 cells into athymic nude mice displayed attenuated tumor growth compared with that of control cells, accompanying decreased protein expression of STAT3 and its target genes such as cyclin D1, c-Myc and Bcl-xL in excised tumor tissues. Taken together, these observations suggest that SIRT1 could contribute to growth and proliferation of colorectal cancer partly via STAT3 signaling pathway.

Keywords: Colon cancer; SIRT1; Proinflammatory cytokine; STAT3

2. Introduction

SIRT1 is a class III histone deacetylase that can deacetylate histone and non-histone proteins. Since the role of SIRT1 in lifespan has been first reported (1), the important function of SIRT1 protein in various intracellular events has been reported for over a decade. SIRT1 contributes to inflammation, apoptosis and energy metabolism through deacetylation of its substrates including NF-κB (2), p53 (3) and PGC-1α (4). The role of SIRT1 in cancer, however, is still ambiguous. Both tumor suppressive and oncogenic functions of SIRT1 have been suggested in colorectal cancer (CRC). Sun and colleagues have reported that knockdown of SIRT1 using SIRT1 shRNA or pharmacologic inhibition promoted the invasion of human colon cancer (SW480) cells, which was mediated through upregulation of the miR-15b-5p transcription (5). Thus, SW480 cells stably expressing wild-type SIRT1 and miR-15b-5p showed more enhanced metastatic potential than SW480 cells stably expressing wild-type SIRT1 in the lung of nude mice. In another study, the conditional SIRT1 transgenic mice which overexpress SIRT1 specifically in the gut villi, showed the reduced intestinal tumor formation, which partly depends on inhibition of cell proliferation by deacetylation-dependent inactivation of β-catenin (6). On the other hand, constitutively upregulated SIRT1 was found to be in CRC tissues compared with adjacent normal tissues (7-9) and significantly associated with the lymph node metastasis (10, 11). It has been also reported that mice with intestine-specific

SIRT1 deletion exhibited the reduced size as well as number of tumors *in vivo*. This was attributable to the increased apoptosis in SIRT1-deficient tumors, which may be due to activation of p53 (12). Moreover, ablation of SIRT1 led to the decreased mRNA expression of stemness-associated genes such as Oct4, Nanog, Cripto and Lin28 in human colon cancer (SW620) cells (13), suggesting the oncogenic potential of SIRT1 in CRC.

Signal transducer and activator of transcription 3 (STAT3) is a member of the STAT protein family. In response to cytokines and growth factors, STAT3 is phosphorylated by receptor-associated Janus kinases, thereby forming homo- or heterodimers. This prompts the translocation of STAT3 to the nucleus, resulting in transcription of target genes, such as cyclin D1, c-Myc and VEGF as well as anti-apoptotic proteins Bcl-2, Bcl-XL and Mcl-1. Overexpression of STAT3 is frequently observed in many cancers including breast, colon and skin cancer and correlates with metastatic progression in cancer patients (14-16). In the CT26 allograft-bearing mice, pharmacologic inhibition of STAT3 by nifuroxazide led to the increased tumor infiltration of active $CD8^+$ T cells and the decreased number of M2 macrophages, thereby exerting antitumor activity (17). In addition, mice with a conditional deletion of STAT3 in enterocytes ($STAT3\Delta^{IEC}$ mice) barely developed the adenomas in azoxymethane/dextran sulfate sodium-induced CRC model, and the tumor size of $STAT3\Delta^{IEC}$ mice was smaller than that of wild-type mice, suggesting protumorigenic role of STAT3 in

colitis-associated cancer (18). Therefore, STAT3 has been considered as a therapeutic target for the treatment of CRC.

In the present study, I investigated the effect of SIRT1 deficiency on the mRNA/protein expression of STAT3 and viability, growth, invasion and migration of CRC cells using siRNA strategy.

3. Materials and methods

Reagents

Dulbecco's Modified Eagle's Medium (DMEM), Minimum Essential Medium (MEM), RPMI 1640 medium and fetal bovine serum (FBS) were purchased from Gibco-BRL (by Thermo Fisher Scientific Inc.; Waltham, MA, USA). Cycloheximide (CHX) was supplied by Sigma-Aldrich (St. Louis, MO, USA). Matrigel® Growth Factor Reduced (GFR) Basement Membrane Matrix was obtained from CORNING Incorporated (Bedford, MA, USA).

Cell culture

HCT-116 and SW480 cells were maintained routinely in DMEM, while HCT-15 and DLD-1 cells were grown in RPMI 1640 medium. CCD841CoN cells were maintained routinely in MEM under humidified atmosphere of 5% CO_2/95% air. Each medium contains 10% FBS and an 100 ng/ml antibiotics mixture. All cell lines were obtained from the Korean Cell Line Bank (KCLB)

Immunofluorescent analysis

Human paraffin-embedded colon cancer tissue array with matched adjacent normal colon tissue (US Biomax, Inc., cat. no. CO703; Rockville, MD, USA) was subjected to

deparaffinization with xylene. Following antigen retrieval by heated citrate buffer, sections were permeabilized and blocked according to the standard protocol. After overnight incubation at 4 °C with anti-SIRT1 antibody, the tissue sections were washed with PBS and then labeled with TRITC-conjugated secondary antibody for 1 h at room temperature. The slides were then analyzed under a fluorescent microscope.

Transient transfection with SIRT1 siRNA

Cells were plated in 60-mm dish and grown to 60% confluence. Scrambled or SIRT1 siRNA (20 nM) was transfected into cells with Lipofectamine® RNAiMAX (Invitrogen by Thermo Fisher Scientific Inc.; Waltham, MA, USA) according to the manufacturer's instructions. The target sequences for human SIRT1 siRNA #1 were sense 5'-ACUUUGCUGUAACCCUGUA(dTdT)-3' and antisense 5'-UACAGGGUUACAGCAAAGU(dTdT)-3' and for human SIRT1 siRNA #2 were sense 5′-AGAGUUGCCACCCACACCU(dTdT)-3′, antisense 5′-AGGUGUGGGUGGCAACUCU(dTdT)-3′. After 48 h of transfection, cells were harvested. siRNA oligonucleotides targeting SIRT1 were purchased from Genolution (Seoul, South Korea).

Transient transfection of empty vector (Mock) or expression vector encoding SIRT1

Transient transfection with plasmid vectors was performed using Lipofectamine® 2000 (Invitrogen by Thermo Fisher Scientific Inc.; Waltham, MA, USA) according to the instructions supplied by the manufacturer. After 48 h of transfection, cells were harvested.

MTT assay

For the MTT assay, cells were seeded in 48-well plates at a density of 0.5×10^4 cells/well. After incubation for the indicated periods of time, cells were treated with the MTT solution (final concentration; 1 mg/ml) for 2 h. The resultant formazan crystals formed in intact cells were dissolved in DMSO, and the absorbance at 570 nm was read using a microplate reader.

Wound healing assay

For the wound healing assay, cells were plated into culture-insert (Ibidi; Martinsried, Germany) to make a 500 μm gap. After detaching the insert from a dish, cells were grown for the indicated period of time and then photographed under the microscope.

Invasion assay

For the invasion assay, 4×10^4 cells were added to the top chamber of the transwell. DMEM supplemented with 10% fetal bovine serum was added to the lower chamber. After 48-h incubation, non-invaded cells were removed by scrubbing with cotton swab. Cells on the lower surface of the membrane were fixed with 100% methanol for 2 min and stained with 0.05% crystal violet. The number of invaded cells was counted under a microscope.

Clonogenic assay

For the clonogenic assay, cells were seeded in 6-well plates (200 cells/well) and grown for 14 days. Colonies were stained with 0.05% crystal violet. The number of colonies (more than 50 cells) was counted.

Anchorage-independent growth assay

For the anchorage-independent growth assay, 6-well plates or 60-mm dishes were pre-coated with 0.5% agarose as the bottom layer. On top of the bottom layer, cells mixed with 0.3% agarose were seeded. Cells were maintained in semi-solid medium for 14 days, and colonies were stained with 0.05% crystal violet. The number of colonies larger than 100 μm was counted under a microscope.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from HCT-116 cells by using the TRIzol® reagent (Invitrogen by Thermo Fisher Scientific Inc.; Waltham, MA, USA), and RT-PCR was performed according to the standard protocol. The primers used for the RT-PCR are as follows (forward and reverse, respectively): for *SIRT1*, 5′-GCAACATCTTATGATTGGCAC-3′ (position 620 to 640) and 5′-AAATACCATCCCTTGACCTGAA-3′ (position 891 to 870) with a product size of 272 bp. For *STAT3*, 5′-GGCTGGTAATTTATATAATCCCT-3′ (position 3433 to 3455) and 5′-ACTAAAAGGCCAATACATTACAA-3′ (position 3605 to 3583) with a product size of 173 bp. For *IL-1β*, 5′-GCATCCAGCTACGAATCTCC-3′ (position 229 to 248) and 5′-CCACATTCAGCACAGGACTC-3′ (position 936 to 917) with a product size of 708 bp. For *IL-6*, 5′-GTGTGAAAGCAGCAAAGAGGC-3′ (position 493 to 513) and 5′-CTGGAGGTACTCTAGGTATAC-3′ (position 652 to 632) with a product size of 160 bp. *IL-8*, 5′-ATGACTTCCAAGCTGGCCGTGGCT-3′ (position 154 to 177) and 5′-TCTCAGCCCTCTTCAAAAACTTCT-3′ (position 445 to 422) with a product size of 292 bp. For *GAPDH*, 5′-GCATGGCCTTCCGTGTCCCC-3′ (position 857 to 876) and 5′-CAATGCCAGCCCCAGCGTCA-3′ (position 1072 to 1053) with a product size of 216 bp.

Western blot analysis

Lysates from cells were separated by running through 6-10% sodium dodecyl sulfate polyacrylamide gel and transferred to the polyvinylidene difluoride membranes according to the protocol described elsewhere(19). The membranes were then incubated with primary antibodies against SIRT1, PCNA and actin (Santa Cruz Biotechnology, Inc.; Dallas, TX, USA), STAT3, cyclin D1 and c-Myc (Cell Signaling Technology; Danvers, MA, USA). Blots were washed with tris-buffered saline with 0.1% Tween-20 and then probed with horseradish peroxidase-conjugated secondary antibodies (Pierce Biotechnology; Rockford, IL, USA). The transferred proteins were visualized by using a Western blotting detection reagent kit (AbClon; Seoul, South Korea).

Nude mouse xenograft assay

For the nude mouse xenograft assay, four-week-old male BALB/c nude mice (total 12) were purchased from RaonBio, Inc (Yongin, South Korea) and were housed under climate-controlled conditions (24℃ at 50% humidity) with a 12-h light/12-h dark cycle. After one week of acclimation period, 5×10^6 scrambled or SIRT1 siRNA transfected HCT-116 cells re-suspended in equal volume of phosphate-buffered saline (PBS) and matrigel (total volume of 200 μl) were subcutaneously injected into the flanks of mice to generate colon cancer xenograft tumors ($n = 6$ per group). Tumor volume was regularly measured with digital caliper

and calculated according to the formula, $V= 0.5ab^2$, where ‘a’ is the longest and ‘b’ is the shortest perpendicular diameters. After mice were sacrificed, xenograft tumors were excised and fixed in formalin for further analysis. All experimental protocols for animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Seoul National University (authorization number: SNU-160912-1).

Statistical analysis

The statistical significance was determined using the Student's *t*-test. Differences between groups were considered statistically significant if $p < 0.05$.

4. Results

SIRT1 is overexpressed in human CRC cell lines as well as human colon tumor tissues

To determine the protein expression of SIRT1 in human CRC tissues compared with adjacent normal colon tissues, immunofluorescent analysis was performed on human CRC tissue microarray. The data revealed that 27 out of 35 samples (77.14%) showed upregulation of SIRT1 in human colon tumor tissues compared with that of corresponding adjacent normal colon tissues, and representative images of immunofluorescent staining of SIRT1 are shown in **Fig. 2-1A**. Likewise, the protein levels of SIRT1 were upregulated in the majority of human CRC cell lines we examined compared with normal human colon epithelial (CCD841CoN) cells (**Fig. 2-1B**). In agreement with these findings, HCT-116, HCT-15 and DLD-1 cells, which express high levels of SIRT1, have short doubling-time, whereas SW480 cells, which display a relatively lower level of SIRT1 show a moderate growth(20).

Knockdown of SIRT1 inhibits cell viability, migration, neoplastic growth and invasion of human CRC cell lines

To verify the role of SIRT1 in viability and migration of colon cancer cells, SIRT1 was silenced in human CRC cell lines by transfection with SIRT1-specific small interfering RNA (siRNA). As illustrated in **Fig. 2-2A**, knockdown of SIRT1 decreased the viability of CRC

cell lines we tested as determined by the MTT assay. In addition, in the wound healing assay, cells transfected with SIRT1 siRNA showed a slower migration capacity compared with those transfected with scrambled siRNA (**Fig. 2-2B**). Additionally, SIRT1 depletion inhibited invasion of HCT-116 cells (**Fig. 2-2C**) as well as colony formation in the clonogenic assay (**Fig. 2-2D**) and the anchorage-independent growth assay (**Fig. 2-2E**).

Knockdown of SIRT1 decreases the transcription of proinflammatory cytokines

The growth and migration of tumor cells are favored by proinflammatory cytokines secreted by a broad range of cells including tumor cells. Some oncogenic proteins can mediate or directly participate in transcription of proinflammatory cytokines, thereby stimulating the proliferation and migration of tumor cells. Based on these notion, we examine the possible involvement of cytokine production in oncogenic function of SIRT1. As shown in **Fig. 2-3A,** silencing of SIRT1 led to decreased mRNA levels of proinflammatory cytokines involved in growth and proliferation of colon cancer cells, such as interleukin (IL)-1β, 6 and 8. In parallel with this finding, HCT-116 cells expressing SIRT1 exhibited enhanced capacity to produce IL-1β, IL-6 and IL-8 (**Fig. 2-3B**). These findings suggest that SIRT1 positively regulates the proinflammatory cytokines, particularly IL-1β, IL-6 and IL-8 at the transcriptional level.

Knockdown of SIRT1 decreases the STAT3 protein stability

Because IL-6 and 8 have been known as target genes of STAT3, we next examine whether SIRT1 deficiency could affect the expression of STAT3. When HCT-116 cells were transiently transfected with SIRT1 siRNA, mRNA expression of STAT3 remained unchanged by knockdown of SIRT1 (**Fig. 2-4A**). However, depletion of SIRT1 resulted in the time-dependent inhibition of STAT3 as measured by Western blot analysis (**Fig. 2-4B**). As knockdown of SIRT1 decreased the protein expression of STAT3 without affecting its mRNA expression, I hypothesized that SIRT1 may positively regulate the STAT3 protein stability. When HCT-116 cells were incubated with cycloheximide (CHX), a protein translation inhibitor, cells transfected with SIRT1 siRNA displayed significantly shortened half-life of STAT3 (**Fig. 2-4C**), suggesting the possibility of STAT3 protein stabilization by SIRT1.

Attenuation of tumor growth in vivo by knockdown of SIRT1

To further evaluate the effect of SIRT1 on tumor formation *in vivo*, a xenograft mouse model was employed using subcutaneous injection of control or SIRT1-silenced cells. The representative image of the excised tumors at three weeks after inoculation is presented in **Fig. 2-5A**. The result indicated that the SIRT1 siRNA group exhibited a lower tumor growth rate than the scrambled siRNA group (**Fig. 2-5B**). Silencing of SIRT1 resulted in a significant

decrease of the tumor volume [mean ± SD (mm^3); 410.29 ± 297.343 (scrambled siRNA) vs 94.63 ± 40.37 (SIRT1 siRNA)] (**Fig. 2-5C**) and the tumor weight [mean ± SD (mg); 333.33 ± 287.52 (scrambled siRNA) vs 66.67 ± 51.64 (SIRT1 siRNA)] (**Fig. 2-5D**) as measured at the time of excision. As depicted in **Fig. 2-5E,** the hematoxylin and eosin (H&E) stained sections of resected tumor tissues showed that polygonal or spindle-shaped tumor cells with hyperchromatic nuclei were less frequent in SIRT1-silenced xenograft tumor compared with those observed in control xenograft tumor. Immunohistochemical analysis also showed a relatively weak brown nuclear staining of proliferating cell nuclear antigen (PCNA) in SIRT1-silenced xenograft tumor, indicative of reduced cellular proliferation (**Fig. 2-5F**). Moreover, knockdown of SIRT1 was associated with decreased protein levels of other cellular proliferation indices, such as cyclin D1, c-Myc and Bcl-xL (**Fig. 2-5G**). Thus, the results from the xenograft mouse model parallel those observed in the *in vitro* assays shown in **Fig. 2-1-4**, corroborating the oncogenic function of SIRT1 in the progression of colon cancer.

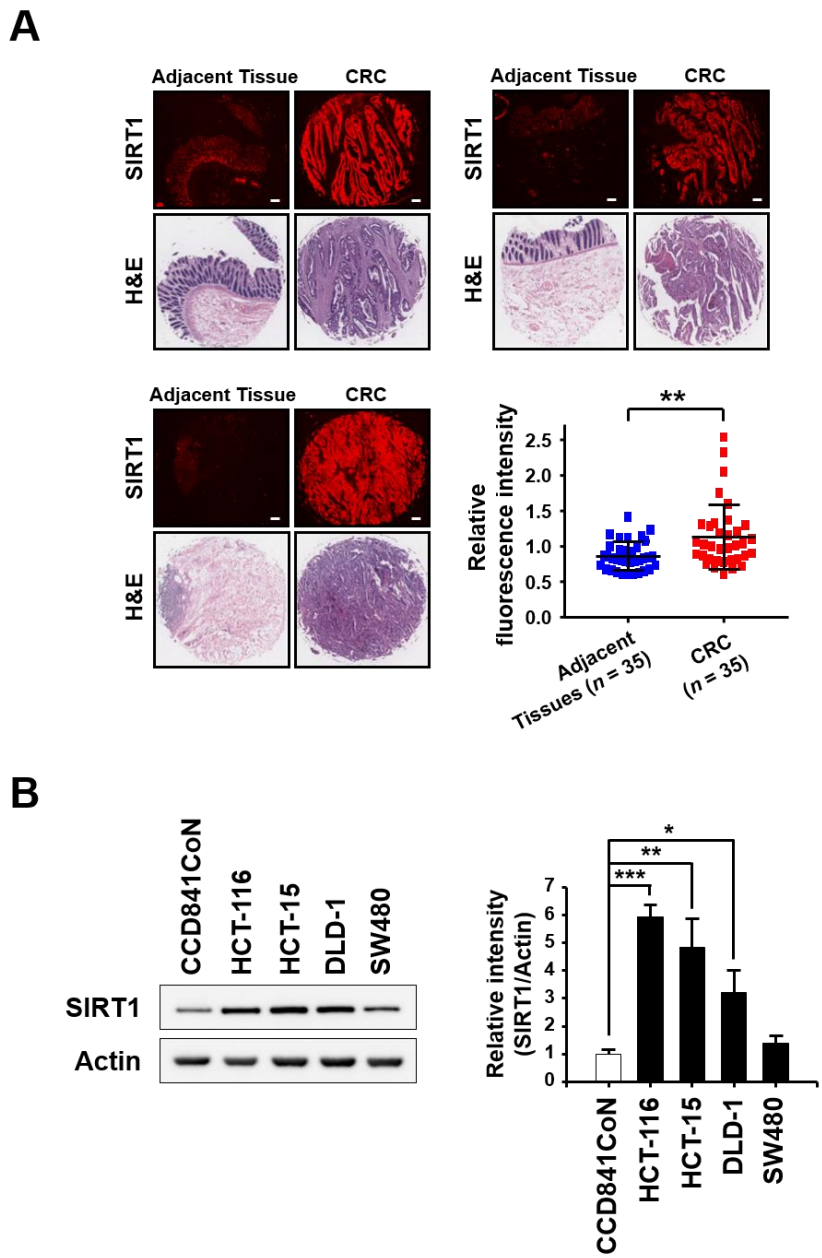
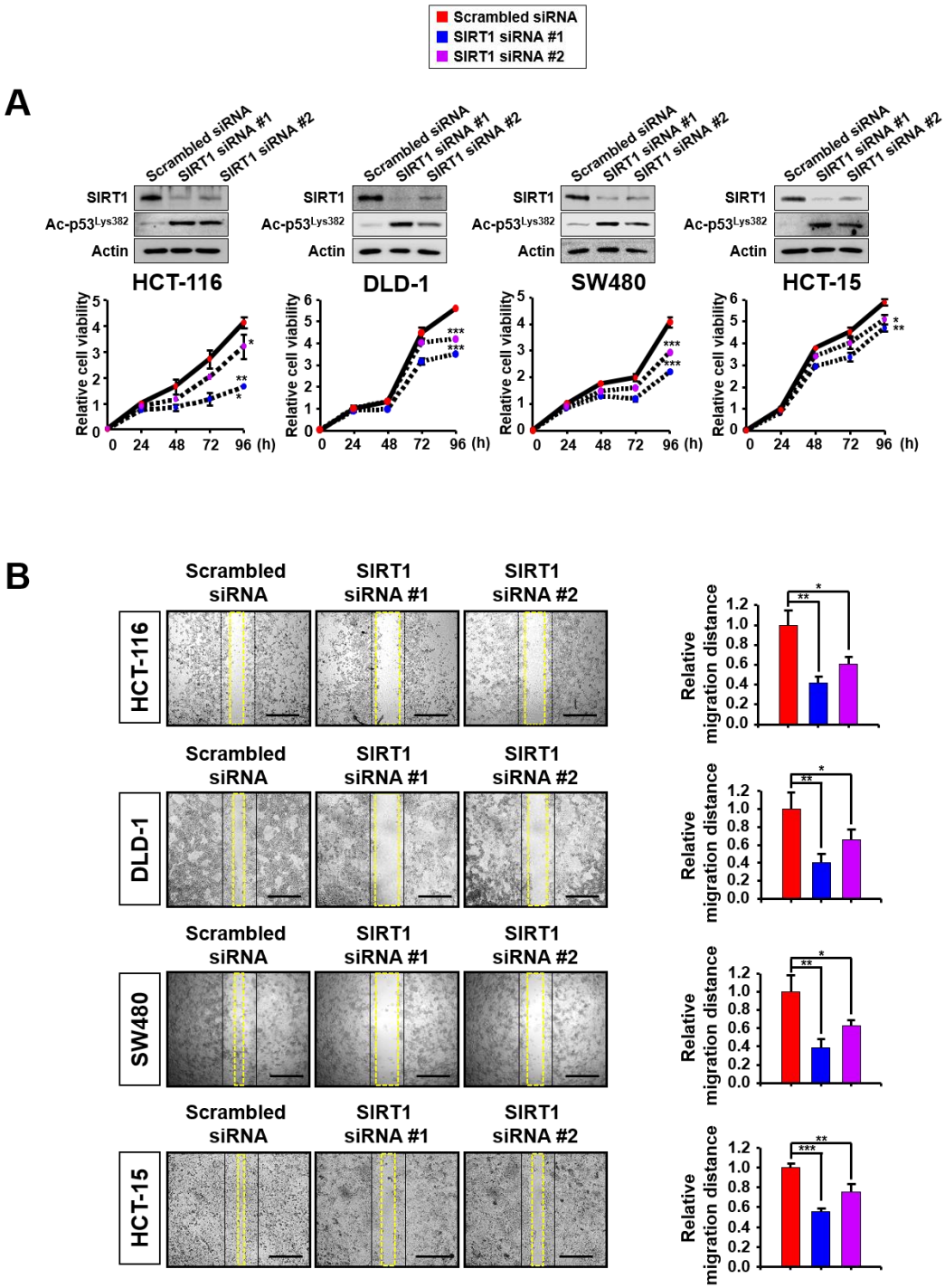


Fig. 2-1. Constitutively elevated expression of SIRT1 in several human CRC cell lines as well as colon adenocarcinoma tissues. (A) Immunofluorescent staining of samples from a colon cancer tissue array with matched adjacent normal colon tissues was performed using

anti-SIRT1 antibody. Surrounding normal colon tissues is further away from 1.5 cm of the rim of tumor, which was determined by a pathologist under microscope to be “normal”. The fluorescent intensity was analyzed by the image processing program Image J and results are shown as the mean ± S.D. of thirty-five samples, $^{**}p < 0.01$. Images of H&E stained tissue sections were provided by US Biomax Inc. Each scale bar represents 200 μm. (B) The basal levels of SIRT1 in different human CRC cell lines (HCT-116, HCT-15, DLD-1 and SW480) as well as a normal human colon epithelial CCD841CoN cells were assessed by Western blot analysis. The values are presented as the mean ± S.D. of three independent experiments, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.


Scrambled siRNA
SIRT1 siRNA #1
SIRT1 siRNA #2
A
SIRT1
Ac-p53Lys382
Actin
HCT-116
DLD-1
SW480
HCT-15
Relative cell viability
0 24 48 72 96 (h)
B
Scrambled siRNA
SIRT1 siRNA #1
SIRT1 siRNA #2
HCT-116
DLD-1
SW480
HCT-15
Relative migration distance

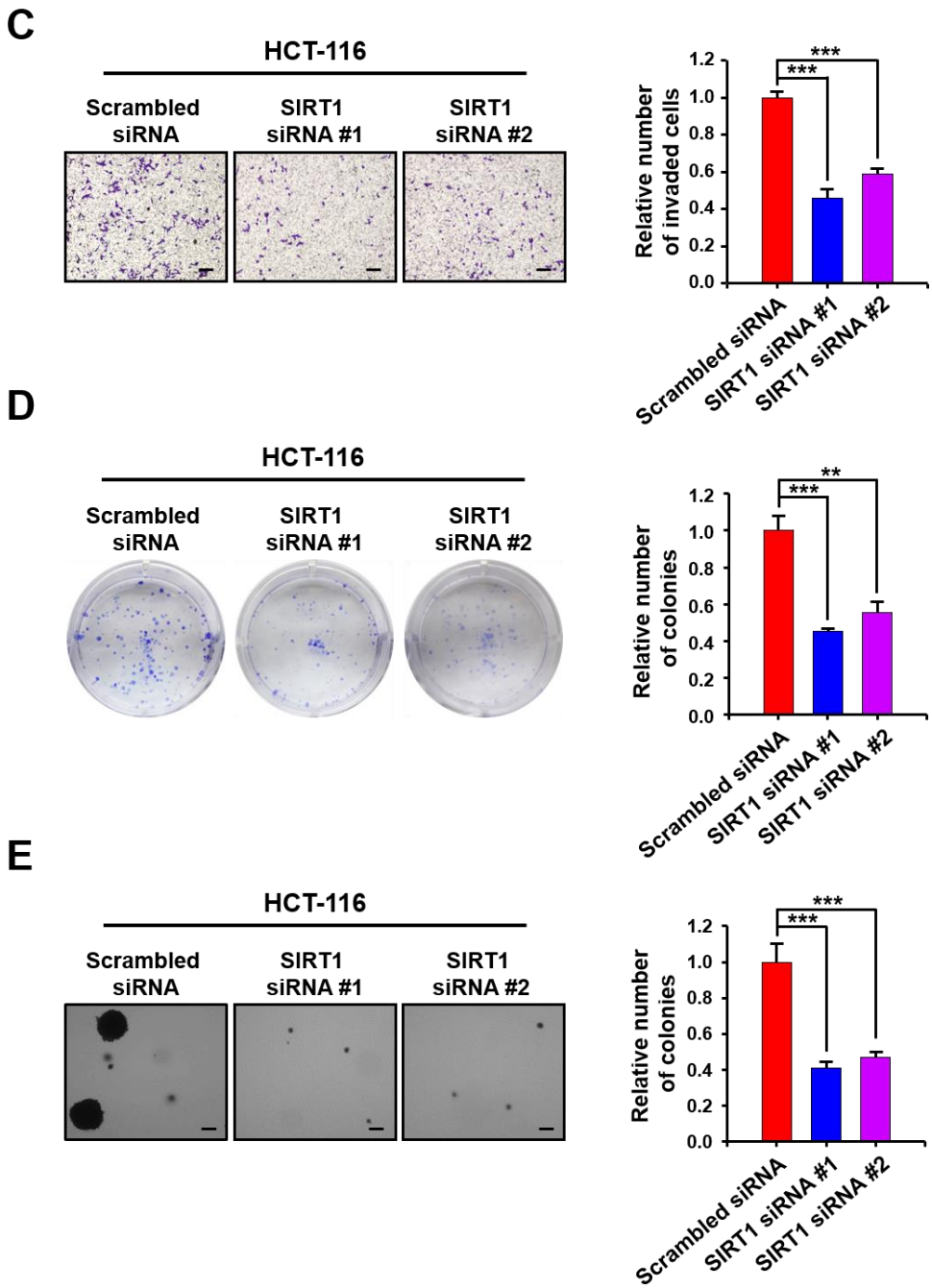


Fig. 2-2. Effects of SIRT1 silencing on viability, migration, invasion and colony formation of CRC cell lines. (A) Human CRC cell lines were transiently transfected with

scrambled or SIRT1 siRNA for 48 h. The cell viability was examined by the MTT assay. The results are shown as the mean ± S.D. of triplicates, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. (B) For the wound healing assay, human CRC cell lines were transiently transfected with scrambled or SIRT1 siRNA for 48 h and were then plated into culture-insert (Ibidi). After 48-h incubation, cell migration was assessed by measuring the wound size under a microscope. Each scale bar represents 500 μm. The values are presented as the mean ± S.D. of three independent experiments, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. (C) For the invasion assay, HCT-116 cells were transiently transfected with scrambled or SIRT1 siRNA for 48 h and were then seeded into the matrigel chambers. Cells invaded through the matrigel-coated membranes were stained with 0.05% crystal violet. Each scale bar represents 200 μm. The results are shown as the mean ± S.D. of three independent experiments, ***$p < 0.001$. (D) Clonogenic efficiency of HCT-116 cells transfected with scrambled or SIRT1 siRNA was measured as described in Materials and Methods. Cells were grown for 14 days, and colonies were stained with 0.05% crystal violet. The data are presented as the mean ± S.D. of triplicates, **$p < 0.01$, ***$p < 0.001$. (E) Colony-forming ability of HCT-116 cells transfected with scrambled or SIRT1 siRNA was assessed by the anchorage-independent growth assay. Cells were maintained in semi-solid medium for 14 days and colonies were stained with 0.05% crystal violet. The number of colonies with a diameter greater than 100 μm was counted under a

microscope. Each scale bar represents 200 μm. The values are presented as the mean ± S.D. of triplicates, ***$p < 0.001$.

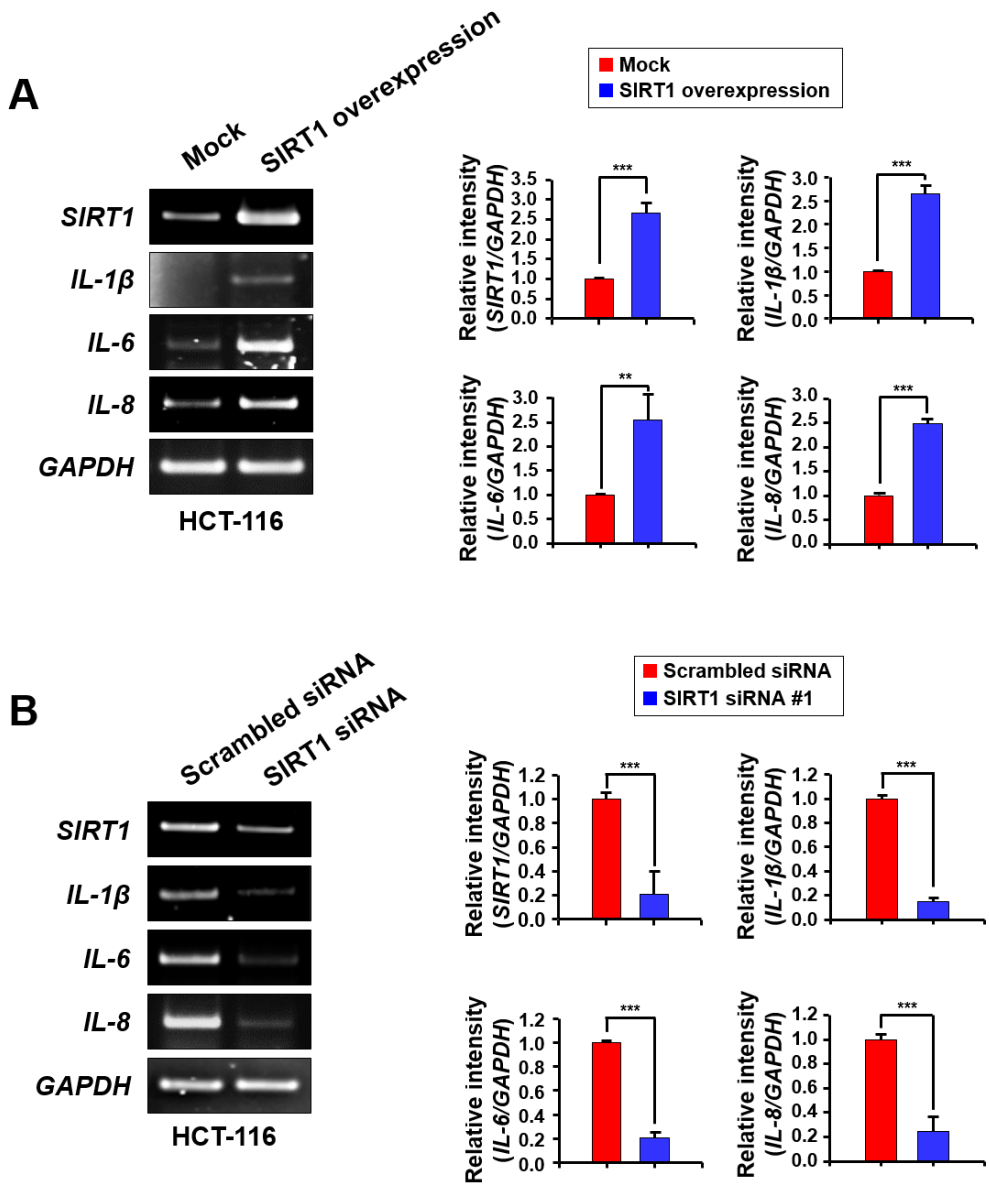


Fig. 2-3. Positive regulation of proinflammatory cytokines by SIRT1. (A) HCT-116 cells were transiently transfected with an empty vector (Mock) or expression vector encoding SIRT1 for 48 h. The mRNA levels of SIRT1, IL-1β, 6 and 8 were examined by RT-PCR. The results are shown as the mean ± S.D. of three independent experiments, $^{**}p < 0.01$, $^{***}p <$

0.001. (B) HCT-116 cells were transiently transfected with scrambled or SIRT1 siRNA for 48 h. The mRNA levels of SIRT1, IL-1β, 6 and 8 were examined by RT-PCR. The data are presented as the mean ± S.D. of triplicates, ***$p < 0.001$.

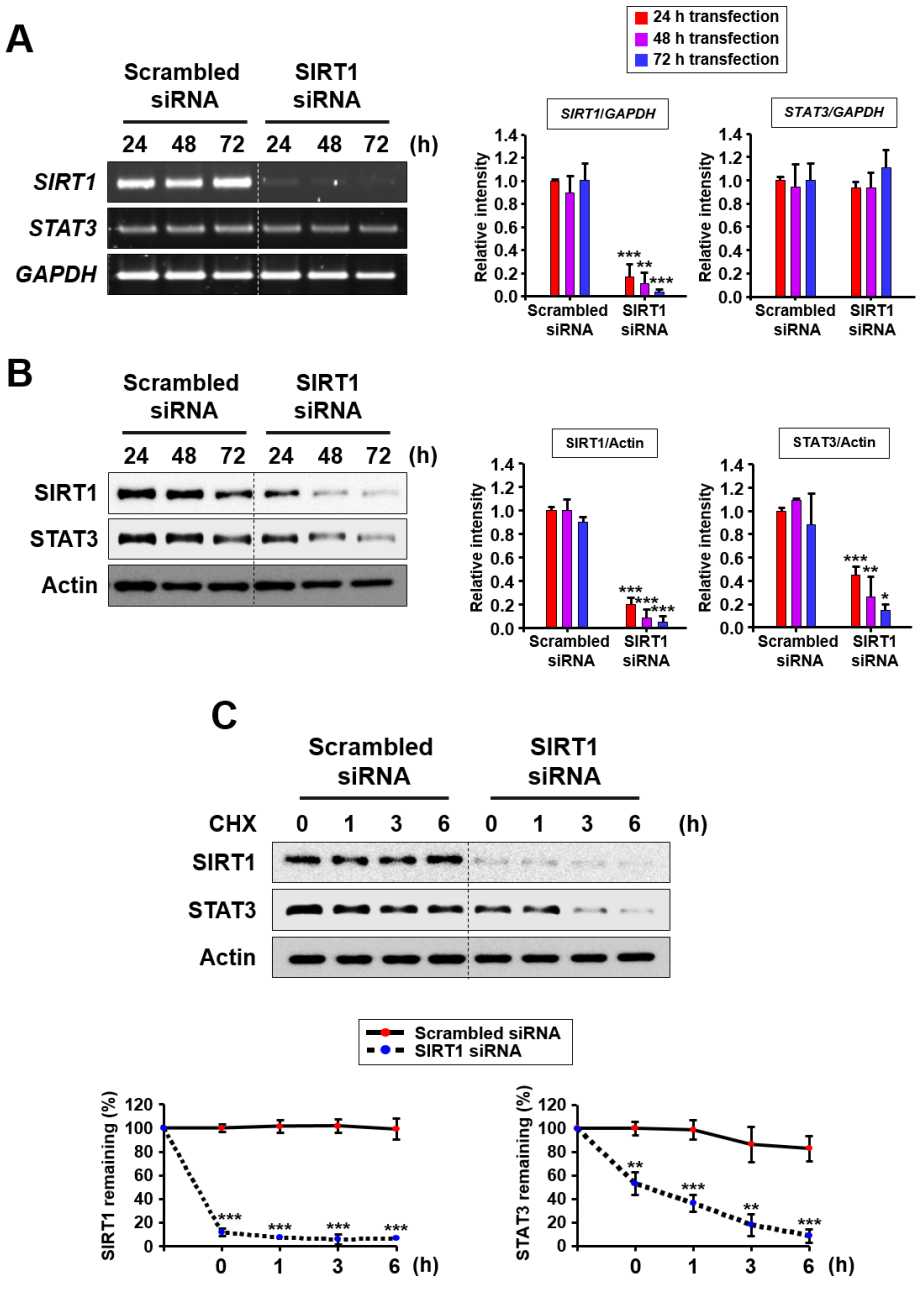
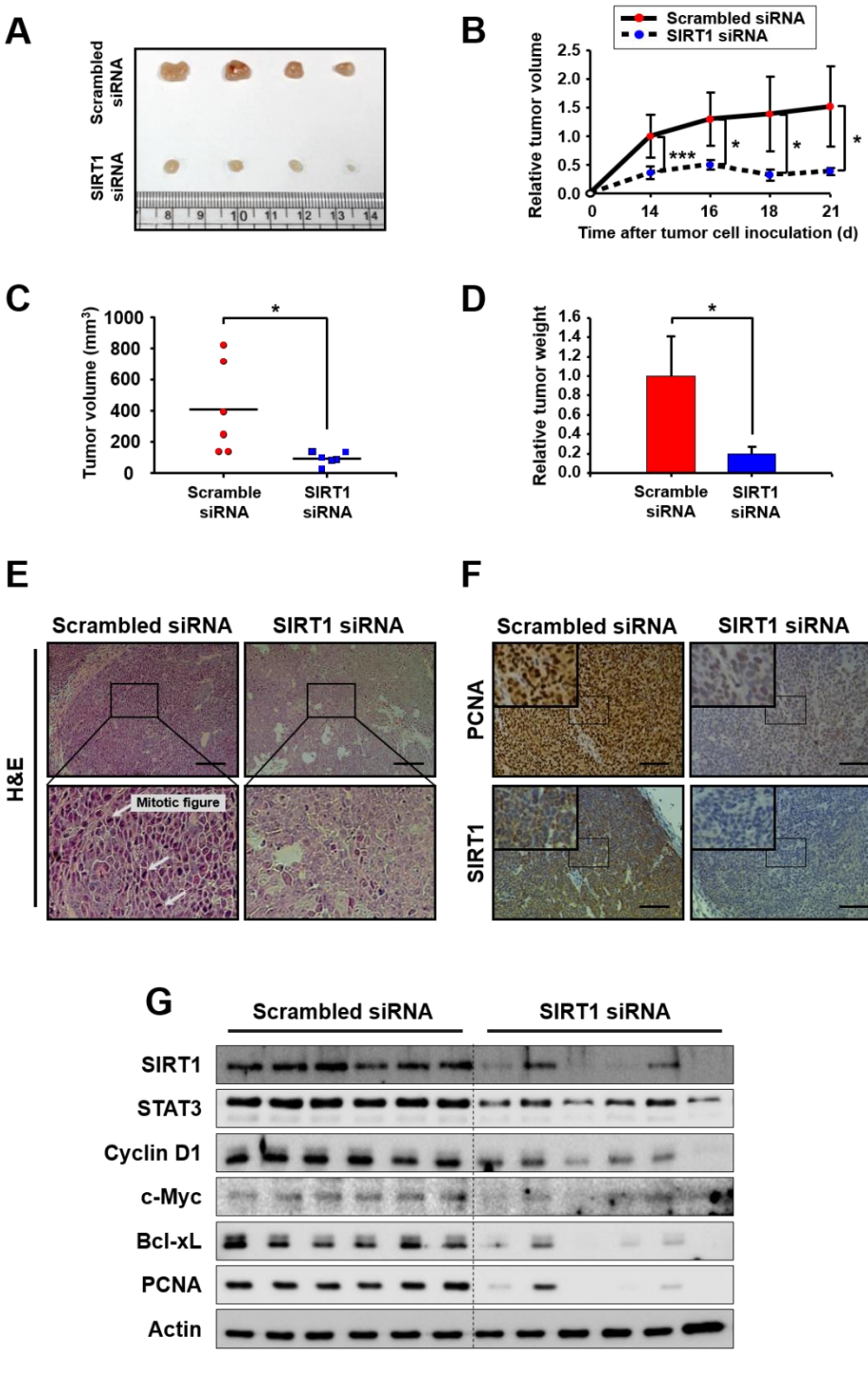


Fig. 2-4. Posttranslational regulation of STAT3 by SIRT1. (A and B) HCT-116 cells were transiently transfected with scrambled or SIRT1 siRNA for the indicated periods of time. The

mRNA (A) and protein (B) levels of SIRT1 or STAT3 were measured by RT-PCR and Western blot analyses, respectively. GAPDH and Actin were used as an equal loading control for normalization. The values are resented as the mean ± S.D. of three independent experiments, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. (C) HCT-116 cells were treated with cycloheximide (CHX; 71.07 μM) for indicated time periods following transfection with scrambled or SIRT1 siRNA, and the protein level of SIRT1 remaining at each time point was determined by immunoblot analysis. The results are shown as the mean ± S.D. of three independent experiments, **$p < 0.01$, **$p < 0.01$, ***$p < 0.001$.


A
Scrambled siRNA
SIRT1 siRNA
B
Scrambled siRNA
SIRT1 siRNA
Relative tumor volume
Time after tumor cell inoculation (d)
C
Tumor volume (mm3)
Scramble siRNA
SIRT1 siRNA
D
Relative tumor weight
Scramble siRNA
SIRT1 siRNA
E
Scrambled siRNA
SIRT1 siRNA
H&E
Mitotic figure
F
Scrambled siRNA
SIRT1 siRNA
PCNA
SIRT1
G
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c-Myc
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Actin

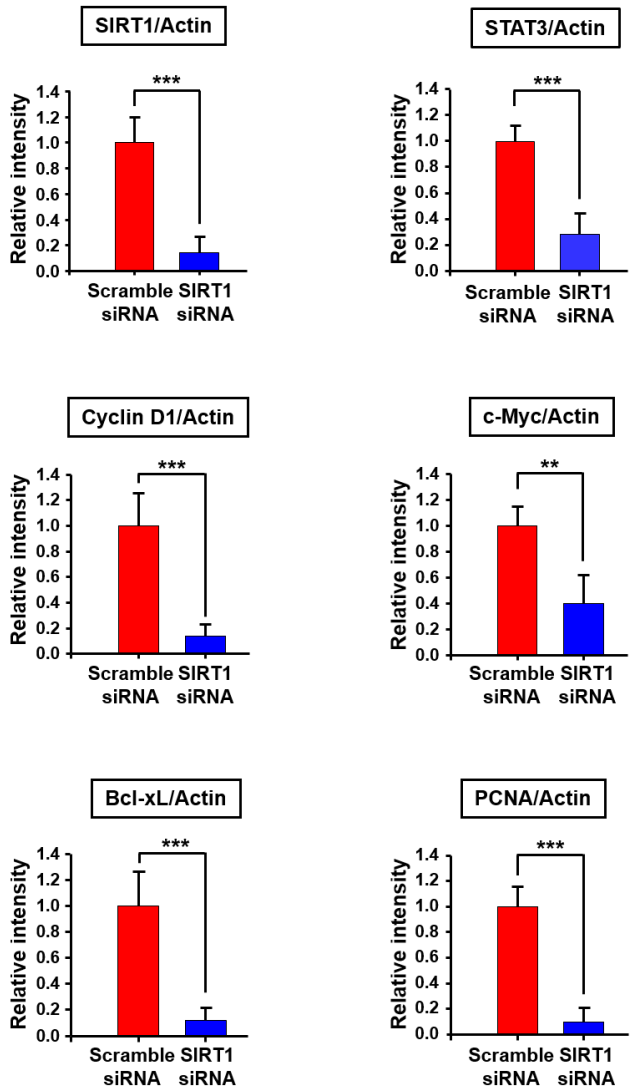

Fig. 2-5. Effects of SIRT1 knock-down on tumor growth in a human colon cancer xenograft model in athymic nude mice. (A) HCT-116 cells were transfected with scrambled or SIRT1 siRNA and 5 × 10^6 cells were injected subcutaneously into the flanks of BALB/c nude mice. Photograph of the xenograft tumors excised from mice at the end of the experiment (day 21). (B) The tumor volume was determined by using the formula, V= 0.5 × longest

diameter × (shortest diameter)^2. The results are shown as the mean ± S.D. of six xenografts for each group, *$p < 0.05$, ***$p < 0.001$. (C) The tumor volume of the mice at the end of the experiment. The data are presented as the mean ± S.D., *$p < 0.05$. (D) The tumor weight of the mice at the end of the experiment. The results are expressed as the mean ± S.D., *$p < 0.05$. (E) Histological features were evaluated by H&E staining in sections from resected xenograft (magnification, × 10). Each scale bar represents 200 μm. (F) Immunohistochemical analysis was performed with sections of excised xenograft using anti-PCNA and SIRT1 antibodies. Each scale bar represents 200 μm. (G) The protein levels of SIRT1, STAT3, cyclin D1, c-Myc and PCNA in resected tumor tissues from mice bearing SIRT1-knockdowned HCT-116 cells were determined by immunoblot analysis. The values are presented as the mean ± S.D. of six samples, **$p < 0.01$, ***$p < 0.001$.

5. Discussion

CRC is the most frequently diagnosed cancer and the leading cause of cancer-related deaths in both male and female worldwide. It is well documented that patients with inflammatory bowel disease have a higher risk of developing CRC, suggesting a close connection between inflammation and CRC. Even after the development of CRC, proinflammatory conditions surrounding the tumor cells can accelerate the tumor progression, invasion and metastasis.

SIRT1 has been reported to possess anti-inflammatory activity, thus its activation has been considered to be beneficial for inflammation-mediated diseases. Indeed, depletion of SIRT1 resulted in increased mRNA expression of IL-1β, IL-6 and TNF-α in LPS-stimulated RAW264.7 macrophages. However, pretreatment of cells with SRT1720, an activator of SIRT1, remarkably suppressed LPS-induced activation of RelA/p65 subunit of NF-κB via deacetylation, indicating anti-inflammatory property of SIRT1 is achieved by inhibition of NF-κB, which is responsible for transcription of the above proinflammatory cytokines (21). Furthermore, activation of SIRT1 by resveratrol, a well-known natural activator, inhibited the production of TNF-α-induced IL-1β and IL-6 in mouse embryonic (3T3/NIH) fibroblasts, which is mediated through deacetylation and subsequent inactivation of p65 (22). However, conflicting observations also exist showing the proinflammatory activity of SIRT1. It has been suggested that SIRT1 positively regulates the production of IL-6 and IL-8 in rheumatoid

arthritis synovial fibroblasts (RASFs) and TNF-α in monocytes, thereby inhibiting apoptosis and facilitating chronic inflammation. This SIRT1-dependent increase of proinflamatory mediators was significantly abrogated by inhibition of NF-κB by sc-514, an inhibitor of IKKβ, suggesting dependency of SIRT1 on NF-κB pathway to exert proinflammatory and anti-apoptotic activity (22). Likewise, SIRT1-overexpressing mouse B lymphocyte (BaF3) cells exhibited the activation of p65 through phosphorylation and subsequent increase in IL-1β, IL-6 and TNF-α at the transcriptional level (23). In the present study, along with the latter observations showing the proinflammatory property of SIRT1, silencing of SIRT1 reduced the proinflammatory mediators, IL-1β, IL-6 and IL-8. This could be one possible reason for restrained growth and migration of human CRC cell lines by knockdown of SIRT1. However, the molecular mechanisms by which SIRT1 positively modulates these proinflammatory molecules remain to be elucidated. Even though NF-κB is closely linked to SIRT1 relative to the anti-inflammatory or proinflammatory property of SIRT1, the facts that STAT3 is engaged in transcription of IL-6 and IL-8 and constitutively activated STAT3 is frequently observed in CRC cells prompted me to investigate the possibility of STAT3 regulation by SIRT1.

Elevated levels of STAT3 are significantly associated with lymph node metastasis in CRC patients (24) and adverse host inflammatory responses in patients undergoing CRC resection (25). Blockade of STAT3 by dominant-negative STAT3 variant slowed down the development

of CRC-derived xenograft tumors, indicating the contribution of STAT3 to colorectal tumor growth (26). SIRT1 has been known to counteract the activation of STAT3 via deacetylation at the lysine 685 residue, thus considered to have anti-tumorigenic activity in gastric and lymphoid malignancies (27, 28). According to recent findings, however, SIRT1 is upregulated in all stages of gastric cancer compared with normal gastric mucosa, and coordinated high expression of SIRT1 and STAT3 is associated with poor overall survival for advanced gastric cancer patients (29). With regard to CRC, positive relationship or cooperation between SIRT1and STAT3 has not yet been suggested.

In the current study, I noticed that the expression of STAT3 is positively related to that of SIRT1 at the protein level, suggesting a possibility of STAT3 protein stabilization by SIRT1. Despite its undoubted function in neoplastic transformation and development of cancer, much less is known about regulation of STAT3 protein stability. It has been known that the level of STAT3 is tightly controlled by its endogenous inhibitory proteins, such as protein inhibitor of activated STAT 3 (PIAS3). PIAS3 blocked the DNA-binding activity of STAT3 and subsequently inhibited the expression of STAT3 target genes (30). Notably, PIAS3 can function as E3 ligases, thus it posttranslationally attaches the small ubiquitin-related modifier (SUMO) to target proteins, resulting in its degradation. Likewise, constitutive

photomorphogenic 1 (COP1) is considered as a putative E3 ligase for STAT3 to induce ubiquitin-dependent degradation of STAT3 in human prostate cancer (DU145) cells.

Considering that both acetylation and ubiquitination occur at the lysine residue of proteins, the interplay between the two lysine modifications implies acetylation-dependent protein stabilization and deacetylation-dependent protein degradation. Based on these notions, I hypothesized that SIRT1 may indirectly regulate the STAT3 protein stability through deacetylation-dependent breakdown of negative regulator of STAT3, such as PIAS3 and COP1.

Taken together, SIRT1 can promote the viability, migration, invasion and growth of colon cancer cells by enhancing the production of proinflammatory cytokines, which is mediated through positive regulation of STAT3 protein stability by SIRT1. This study therefore provides the basis for SIRT1 and STAT3 as possible therapeutic targets for treatment of CRC.

6. References

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Chapter III

Curcumin suppresses oncogenicity of human colon cancer cells by covalently modifying the cysteine 67 residue of SIRT1

1. Abstract

SIRT1, an NAD^+-dependent histone/protein deacetylase, has diverse physiological actions. Recent studies have demonstrated that SIRT1 is overexpressed in colorectal cancer, suggesting its oncogenic potential. However, the molecular mechanisms by which overexpressed SIRT1 induces the progression of colorectal cancer and its inhibition remain largely unknown. Curcumin (diferuloymethane), a major component of the spice turmeric derived from the plant *Curcuma longa* L., has been reported to exert chemopreventive and anti-carcinogenic effects on colon carcinogenesis. In the present study, we found that curcumin reduced the expression of SIRT1 protein without influencing its mRNA expression in human colon cancer cells, suggesting posttranslational regulation of SIRT1 by this phytochemical. Notably, ubiquitination and subsequent proteasomal degradation of SIRT1 were induced by curcumin treatment. Results of nano-LC-ESI-MS/MS revealed the direct binding of curcumin to cysteine 67 of SIRT1. In line with this result, the protein stability and clonogenicity of a mutant SIRT1 in which cysteine 67 was substituted by alanine were unaffected by curcumin. Taken together, these observations suggest that curcumin facilitates the proteasomal degradation of oncogenic SIRT1 through covalent modification of SIRT1 at the cysteine 67 residue.

Keywords: Colorectal cancer; SIRT1; Curcumin; Tetrahydrocurcumin; Thiol modification

2. Introduction

Colorectal cancer (CRC) is the most common malignancy worldwide. It is the third leading cause of cancer-related death in both men and women in the United States (1). It has been widely believed that frequent consumption of Western diet, especially red meat and saturated fats, increases the risk of CRC. In contrast, the incidence of CRC in India is relatively low. Epidemiologic studies suggest that dietary phytochemicals, such as curcumin abundant in Indian food, may be responsible for the low risk of intestinal cancer in India (2).

Curcumin, a yellow pigment present in the rhizome of turmeric (*Curcuma longa* L., Zingiberaceae) used in preparing the curry powder, is one of the most extensively investigated phytochemicals in regard to chemopreventive and antitumorigenic potential. Curcumin modulates the expression or activity of multiple proteins involved in cellular signal transduction pathways. It has been shown to block the tumor necrosis factor-alpha-induced nuclear translocation and DNA binding of NF-κB in human myelomonoblastic leukemia (ML-1a) cells (3). Curcumin also suppressed the expression of the c-Fos subunit of AP-1 and its DNA binding activity in HPV-18-positive HeLa cells (4).

The SIRT protein family of NAD^+-dependent class III histone deacetylase consists of seven isoforms (SIRT1-7). Among these, SIRT1 was first reported to extend the life span of budding yeast (5). Subsequent studies demonstrated diverse physiological actions of SIRT1,

such as metabolic regulation, differentiation and stress response. However, the role of SIRT1 in tumorigenesis is still controversial. It has been shown that SIRT1 exerts tumor suppressive effects by deacetylating the lysine 310 residue of RelA/p65 subunit of NF-κB, a tumor promoting transcription factor often overexpressed in cancer cells. This leads to decreased production of the anti-apoptotic cIAP-2 gene, sensitizing human non-small cell lung cancer (NCI-H1299) cells to apoptosis (6). Moreover, SIRT1 reduces TGF-β-driven epithelial-to-mesenchymal transition of V12H-Ras transformed human breast epithelial (HMLER) cells by deacetylating Smad4 and repressing its transcriptional activity toward the MMP7. As a result, the epithelial marker E-cadherin is less susceptible to cleavage from the cell surface (7). On the other hand, there are some reports suggesting that SIRT1 may act as a tumor promoter via deacetylation and inactivation of the p53 suppressor protein. Thus, deacetylation of p53 at lysine 382 by SIRT1 causes attenuation of p53-dependent apoptosis in response to DNA damage in human non-small cell lung cancer (NCI-H1299) and human breast cancer (MCF-7L) cells (8). Further, it has been suggested that SIRT1 promotes the development of prostate cancer through deacetylation-dependent inactivation of forkhead box protein O1 (FOXO1), a transcription factor involved in cell cycle arrest, apoptosis and detoxification of reactive oxygen species (9).

Our previous studies have revealed that SIRT1 is highly expressed in human colon tumor

tissues compared with adjacent normal tissues (10), which is implicated in the progression of CRC (11). Here, we report that curcumin directly binds to SIRT1 and decreases its stability in human colon cancer (HCT-116) cells, thereby suppressing its oncogenicity.

3. Materials and methods

Reagents

Curcumin was purchased from LKT Laboratories, Inc (St. Paul, MN, USA). Tetrahydrocurcumin prepared by a conventional catalytic hydrogenation of curcumin was kindly provided by Prof. Jeewoo Lee of Seoul National University. Dulbecco's Modified Eagle's Medium (DMEM), Minimum Essential Medium (MEM), RPMI 1640 medium and fetal bovine serum (FBS) were purchased from Gibco-BRL (by Thermo Fisher Scientific Inc.; Waltham, MA, USA). MG-132 was obtained from Enzo Life Sciences (Exeter, UK). CNBr-Sepharose 4B was supplied by Amersham Biosciences (Buckinghamshire, UK). Matrigel® Growth Factor Reduced (GFR) Basement Membrane Matrix was obtained from CORNING Incorporated (Bedford, MA, USA).

Cell culture

HCT-116 and SW480 cells were maintained routinely in DMEM, while HCT-15, DLD-1 and MCF-7 cells were grown in RPMI 1640 medium. CCD841CoN cells were maintained routinely in MEM under humidified atmosphere of 5% CO_2/95% air. Each medium contains 10% FBS and an 100 ng/ml antibiotics mixture. All cell lines were obtained from Korean Cell

Line Bank (KCLB).

Measurement of SIRT1 catalytic activity

SIRT1 deacetylase activity was assessed by using the fluorometic activity assay kit (Sigma-Aldrich; St. Louis, MO, USA) according to the manufacturer's protocol. Samples were transferred to the Costar 96-well dark plate, and the deacetylation-dependent fluorescent signal was detected by using a fluorescent reader with 360 nm excitation and 460 nm emission wavelengths.

Western blot analysis

Lysates from cells were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to the polyvinylidene difluoride membranes according to the protocol described elsewhere (12). The membranes were then incubated with primary antibodies against SIRT1, His tag, actin (Santa Cruz Biotechnology, Inc.; Dallas, TX, USA), ubiquitin (Life Technolgies by Thermo Fisher Scientific Inc.; Waltham, MA, USA), acetyl-p53 (Cell Signaling Technology; Danvers, MA, USA) and FLAG (Sigma-Aldrich; St. Louis, MO, USA). Blots were washed with tris-buffered saline with 0.1% Tween-20 and then

probed with horseradish peroxidase-conjugated secondary antibodies (Pierce Biotechnology; Rockford, IL, USA). The transferred proteins were visualized by using a Western blotting detection reagent kit (AbClon; Seoul, South Korea).

Reverse transcription-polymerase chain reaction (RT-PCR) and real-time PCR

Total RNA was isolated from HCT-116, DLD-1, HCT-15, SW480 and MCF-7 cells by using the TRIzol® reagent (Invitrogen by Thermo Fisher Scientific Inc.; Waltham, MA, USA), and RT-PCR was performed according to the standard protocol. The primers used for the RT-PCR are as follows (forward and reverse, respectively): for *SIRT1*, 5′-GCAACATCTTATGATTGGCAC-3′ (position 620 to 640) and 5′-AAATACCATCCCTTGACCTGAA-3′ (position 891 to 870) with a product size of 272 bp. For *GAPDH*, 5′-GCATGGCCTTCCGTGTCCCC-3′ (position 857 to 876) and 5′-CAATGCCAGCCCCAGCGTCA-3′ (position 1072 to 1053) with a product size of 216 bp. For the real-time PCR, a RealHelix™ SYBR Green I qPCR kit (NanoHelix Co., Ltd.; Seoul, South Korea) was used. The primers used for the real-time PCR are as follows: for *SIRT1*, 5'-AGAAGAACCCATGGAGGATG-3' (position 1391 to 1410), and 5'-TCATCTCCATCAGTCCCAAA-3' (position 1504 to 1485) with a product size of 114 bp. For *RPL32*, 5'-CTCTTTCCACGATGGCTTTG-3' (position 437 to 418) and 5'-

GTCAAGGAGCTGGAAGTGCT-3' (position 338 to 357) with a product size of 100 bp. PCR products were measured by 7500 Fast Real-Time PCR System (Applied Biosystems by Thermo Fisher Scientific Inc.; Waltham, MA, USA) using fluorescent SYBR Green I dye.

Immunocytochemical analysis

Cells grown on coverslips were prepared as described previously (13). Cells were then blocked with PBS with Tween-20 containing 5% bovine serum albumin for 1 h at room temperature and incubated with an antibody against SIRT1 overnight. After rinse with PBS, cells were labeled with FITC-conjugated secondary antibody for 1 h at room temperature. Finally, cells were further stained with propidium iodide (PI; Molecular Probes®; Eugene, OR, USA) to visualize the nuclei. The slides were then analyzed under a fluorescent microscope.

Preparation of cytosolic and nuclear proteins

Confluent cells in 100-mm dishes were treated with curcumin and then harvested. According to purpose of the experiment, cells were transiently transfected with FLAG-tagged wild-type (SIRT1-WT) or mutant SIRT1 (SIRT1-C67A) and then harvested. Pellets were suspended in hypotonic lysis buffer to obtain cytosolic extract, and then resulting pellets were suspended

in hypertonic lysis buffer to collect the nuclear extract according to the previously reported procedure (14). Each separately obtained supernatant containing cytosolic or nuclear proteins was stored at -70°C after determination of the protein concentration.

Immunoprecipitation

For ubiquitination of endogenous SIRT1, cells were treated with MG-132 (20 μM) and curcumin (10 μM) for 3 h. For ubiquitination of exogenous SIRT1, cells were transfected with FLAG-SIRT1 and His-ubiquitin plasmids and incubated with MG-132 (20 μM) and curcumin (10 μM) for 3 h. Lysates from cells were prepared as described earlier (14). After resolving the samples by SDS-PAGE, membranes were incubated with primary antibody to His tag.

Wound healing assay

For the wound healing assay, cells were plated into Culture-insert (Ibidi; Martinsried, Germany) to make a 500 μm gap. After detaching the insert from a dish, cells were grown for the indicated period of time and then photographed under the microscope.

Anchorage-independent growth assay

For the anchorage-independent growth assay, 6-well plates or 60-mm dishes were pre-coated with 0.5% agarose as the bottom layer. On top of the bottom layer, cells mixed with 0.3% agarose were seeded. After allowing the 0.3% agarose to solidify, media containing DMSO or curcumin were added to each well or dish. Cells were maintained in semi-solid medium for 14 days, and colonies were stained with 0.05% crystal violet. The number of colonies larger than 100 μm was counted under a microscope.

Preparation of curcumin- or tetrahydrocurcumin-Sepharose 4B beads

For the pull down assay, curcumin- or tetrahydrocurcumin-conjugated Sepharose 4B beads were prepared according to the protocol described by Urusova *et al*. (15). Proteins bound to the beads were analyzed by immunoblotting with anti-SIRT1 or FLAG antibody.

Nude mouse xenograft assay

For the nude mouse xenograft assay, four-week-old male BALB/c nude mice (total 30) were purchased from RaonBio, Inc (Yongin, South Korea) and were housed under climate-controlled conditions (24℃ at 50% humidity) with a 12-h light/12-h dark cycle. After one

week of acclimation period, 5 × 10^6 HCT-116 cells re-suspended in equal volume of phosphate-buffered saline (PBS) and matrigel (total volume of 200 μl) were subcutaneously injected into the flanks of mice to generate colon cancer xenograft tumors. When the tumor size reached approximately 150 mm^3, mice were randomized into control and treatment groups (50 mg/kg and 100 mg/kg of curcumin or 50 mg/kg and 100 mg/kg of tetrahydrocurcumin). Curcumin or tetrahydrocurcumin dissolved in corn oil was peritumorally administered to mice for a total of eight times during three weeks ($n = 6$ per group). Tumor volume was regularly measured with digital caliper and calculated according to the formula, $V= 0.5ab^2$, where 'a' is the longest and 'b' is the shortest perpendicular diameters. After mice were killed, xenograft tumors were excised and fixed in formalin for further analysis. All experimental protocols for animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Seoul National University (authorization number: SNU-160912-1).

Analysis by nano-LC-ESI-MS/MS

Recombinant SIRT1 protein (Active Motif, cat. no. 31340; Carlsbad, CA, USA) at 50 nM in a final volume of 50 μl 20 mM Tris-HCl (pH 8.0) containing 100 mM KCl, 1 mM dithiothreitol (DTT), 0.2 mM EDTA and 20% glycerol was incubated for 2 h at room

temperature in the presence of DMSO or curcumin (10 μM). The products of in-gel digestion of recombinant SIRT1 pre-incubated with or without curcumin were processed according to procedure as previously described (16). The acquired LC-ESI-MS/MS fragment spectra were searched in the BioWorksBrowserTM (version Rev. 3.3.1 SP1; Thermo Fisher Scientific Inc., San Jose, CA, USA) with the SEQUEST search engines against the data in FASTA format generated from SIRT1 (NCBI accession number NM_012238) in National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/).

Site-directed mutagenesis

Mutations in wild-type SIRT1 were introduced by site-directed mutagenesis using a QuikChange® site-directed mutagenesis kit (Stratagene; Cedar Creek, TX, USA) according to the manufacturer's protocol. Mutant strand synthesis reaction was performed by denaturation at 96°C for 45 sec, annealing at 60°C for 45 sec and extension at 72°C for 8 min with 30 cycles using 5.4 kb plasmid template encoding FLAG-SIRT1. Generally, extension time of 1 min/kb is recommended depending on the length of plasmid template and extension time of 8 minutes was set as an optimal condition. The following complementary primer pairs were used: Cys67Ala: sense, 5′-GCGGCGGCCAGGGGCGCCCCGGGTGCGGCGGCG-3′; antisense, 5′-

CGCCGCCGGTCCCCGCGGGGCCCACGCCGCCGC-3′. The DNA sequences of all plasmids were verified by sequencing (Cosmo Genetech, Seoul, South Korea).

Transient transfection of His-Ub, FLAG-SIRT1-WT and FLAG-SIRT1-C67A plasmid

Transient transfection with plasmid vectors was performed using Lipofectamine® 2000 (Invitrogen by Thermo Fisher Scientific Inc.; Waltham, MA, USA) according to the instructions supplied by the manufacturer. After 48 h of transfection, cells were harvested or treated with DMSO or curcumin according to purpose of the experiment.

Statistical analysis

The statistical significance was determined using the Student's *t*-test. Differences between groups were considered statistically significant if $p < 0.05$.

4. Results

Curcumin reduces the expression of SIRT1 protein without influencing its mRNA expression in colon cancer cells

To investigate the ability of curcumin to regulate SIRT1 in colon cancer cells, we first examined whether curcumin could affect the protein expression of SIRT1. When HCT-116 cells were treated with curcumin (1 or 10 μM) for 3 h, the protein level of SIRT1 decreased significantly at the higher concentration (**Fig. 3-1A**). Consistent with this finding, catalytic activity of SIRT1 was gradually diminished upon curcumin treatment (**Fig. 3-1B**). However, curcumin had no significant effect on the protein expression or the catalytic activity of SIRT1 in normal human colon epithelial (CCD841CoN) cells (**Fig. 3-1C** and **D**, respectively). When HCT-116 cells were exposed to 10 μM curcumin for various time periods, there was the time-dependent inhibition of SIRT1 protein expression as measured by Western blot analysis (**Fig. 3-1E**). However, the expression of its mRNA transcript remained unchanged by curcumin treatment as revealed by RT-PCR analysis (**Fig. 3-1F)** This finding was verified by Real-time PCR analysis (**Fig. 3-1G).** Similar observations were made in other human CRC cell lines as well as human breast cancer MCF-7 cells (**Fig. 3-1H**). Immunoblot (**Fig. 3-1I)** and immunocytochemical (**Fig. 3-1J**) analyses further revealed that curcumin markedly reduced the cytosolic accumulation of SIRT1.

Curcumin stimulates ubiquitin-dependent proteasomal degradation, thereby decreasing the protein stability of SIRT1

As curcumin decreased the protein expression of SIRT1 without affecting its mRNA expression, we postulated that curcumin could decrease the SIRT1 protein stability. To test this supposition, HCT-116 cells were pre-incubated with cycloheximide (CHX), a protein synthesis inhibitor, prior to exposure to curcumin. Treatment of HCT-116 cells with curcumin significantly shortened the half-life of SIRT1 (**Fig. 3-2A**), suggesting that curcumin destabilizes SIRT1 by enhancing its degradation rather than inhibiting *de novo* synthesis. Notably, the inhibitory effect of curcumin on SIRT1 protein accumulation was blunted by a proteasome-specific inhibitor MG-132 (**Fig. 3-2B**). MG-132 alone also enhanced the basal level of SIRT1 protein, indicating that proteasomes are required for both steady-state and curcumin-induced degradation of SIRT1. Curcumin-induced proteasomal degradation of SIRT1 was associated with its ubiquitination (**Fig. 3-2C**). Moreover, the ubiquitination of exogenous SIRT1 was also enhanced by curcumin in HCT-116 cells (**Fig. 3-2D**).

The α,β-unsaturated carbonyl group of curcumin plays an important role in reducing the stability of SIRT1 as well as growth of HCT-116 tumor xenografts

Several studies have indicated that anti-inflammatory and anti-carcinogenic properties of curcumin are attributed to its electrophilic α,β-unsaturated carbonyl moiety (17-19). This prompted us to compare curcumin (**Fig. 3-3A, left**) and its non-electrophilic analogue tetrahydrocurcumin (**Fig. 3-3A, right**) for their effects on SIRT1 stability. While curcumin markedly reduced the expression level of SIRT1 protein, tetrahydrocurcumin had no effect (**Fig. 3-3B**). In line with this observation, tetrahydrocutcumin barely induced ubiquitination of SIRT1 (**Fig. 3-3C**). Curcumin has been reported to react directly with intracellular proteins through its α,β-unsaturated carbonyl group (20-23). A pull-down experiment using curcumin- or tetrahydrocurcumin-conjugated Sepharose 4B beads showed that curcumin bound to SIRT1, but tetrahydrocurcumin only weakly interacted with SIRT1 (**Fig. 3-3D**). These findings suggest that the α,β-unsaturated carbonyl group of curcumin is essential for its direct interaction with SIRT1, which appeared to facilitate proteasomal degradation. In parallel with these results, curcumin apparently diminished the anchorage-independent growth (**Fig. 3-3E**) and migration (**Fig. 3-3F**) of HCT-116 cells, whereas tetrahydrocurcumin was not effective. To explore the effects of curcumin and its non-electrophilic analogue on tumor growth *in vivo*, mice bearing HCT-116 xenografts were treated with peritumoral injection (50 or 100 mg/kg) of each compound for a total of eight times during three weeks. As depicted in **Fig. 3-3G**, curcumin is much more effective than tetrahydrocurcumin in retarding tumor growth.

Injection of 50 or 100 mg/kg of curcumin exhibited significant reduction in the tumor volume [mean ± SD (mm^3); 533.12 ± 100.23 (vehicle), 305.52 ± 147.17 (curcumin, 50 mg/kg), 125.50 ± 49.67 (curcumin, 100 mg/kg), 500.18 ± 99.45 (tetrahydrocurcumin, 50 mg/kg), 501.23 ± 123.43 (tetrahydrocurcumin, 100 mg/kg)] (**Fig. 3-3H**) and the tumor weight [mean ± SD; 716.67 ± 75.28 (vehicle) 350.17 ± 176.01 (curcumin, 50 mg/kg), 200.33 ± 89.00 (curcumin, 100 mg/kg), 616.67 ± 160.21 (tetrahydrocurcumin, 50 mg/kg), 600.50 ± 89.45 (tetrahydrocurcumin, 100 mg/kg)] (**Fig. 3-3I**). Histological examination of tumor tissues from the mice treated with curcumin exhibited necrotic cell death (**Fig. 3-3J, upper panels**) whereas those from mice treated with an 100 mg/kg dose of tetrahydrocurcumin showed slight reduction in hyperchromatic tumor cells (**Fig. 3-3K**). Concomitantly, immunohistochemical staining of SIRT1 in the xenograft tumors from the mice treated with curcumin showed a relatively weak intensity compared with those of control or tetrahydrocurcumin group (**Fig. 3-3J, lower panels**). The inhibitory effect of curcumin on SIRT1 protein expression in HCT-116 tumor xenografts was verified by Western blot analysis (**Fig. 3-3L**).

Cysteine 67 of SIRT1 is a putative target for its covalent modification by curcumin

Nucleophilic cysteine thiols are readily attacked by the reactive electrophilic α,β-unsaturated carbonyl group in the Michael addition reaction (24, 25). To verify the possible involvement

of thiol modification of a specific cysteine residue(s) of SIRT1 by curcumin, HCT-116 cells were treated with curcumin in the presence or absence of the thiol reducing agent DTT or *N*-acetyl-L-cysteine (NAC). The result indicated that both DTT and NAC dampened curcumin-mediated suppression of SIRT1 (**Fig. 3-4A** and **B**, respectively). These observations imply that one or more of SIRT1 cysteine residue(s) is/are susceptible to covalent modification by curcumin, which facilitates proteasomal degradation of SIRT1. To further investigate the modifiable residue(s) of SIRT1 by curcumin, recombinant human SIRT1 was incubated with curcumin followed by nano-LC-ESI-MS/MS analysis. The data revealed modification of the cysteine 67 residue by curcumin (**Fig. 3-4C**). A peak of the 755.28 Da molecular mass was detected only in curcumin-treated sample in addition to a peak of 386.15 Da in the control. The difference in masses between the curcumin treated sample and the DMSO treated sample was 368.38, which corresponds to the molecular weight of curcumin. To verify the significance of cysteine 67 in interaction between curcumin and SIRT1, cysteine to alanine substitution mutant (SIRT1-C67A) was generated by site-directed mutagenesis. The resulting mutant SIRT1 construct was transfected into HCT-116 cells. Subcellular localization (**Fig. 3-4D**) and deacetylase activity (**Fig. 3-4E** and **F**) of SIRT1 were not affected by cysteine 67 to alanine substitution. As shown in **Fig. 3-4G**, curcumin failed to bind to SIRT1 mutated at cysteine 67. Consistent with a result in HCT-116 cells, the same mutation (SIRT1-C67A) in

DLD-1 cells also hampered the covalent interaction between curcumin and SIRT1 (**Fig. 3-4H**).

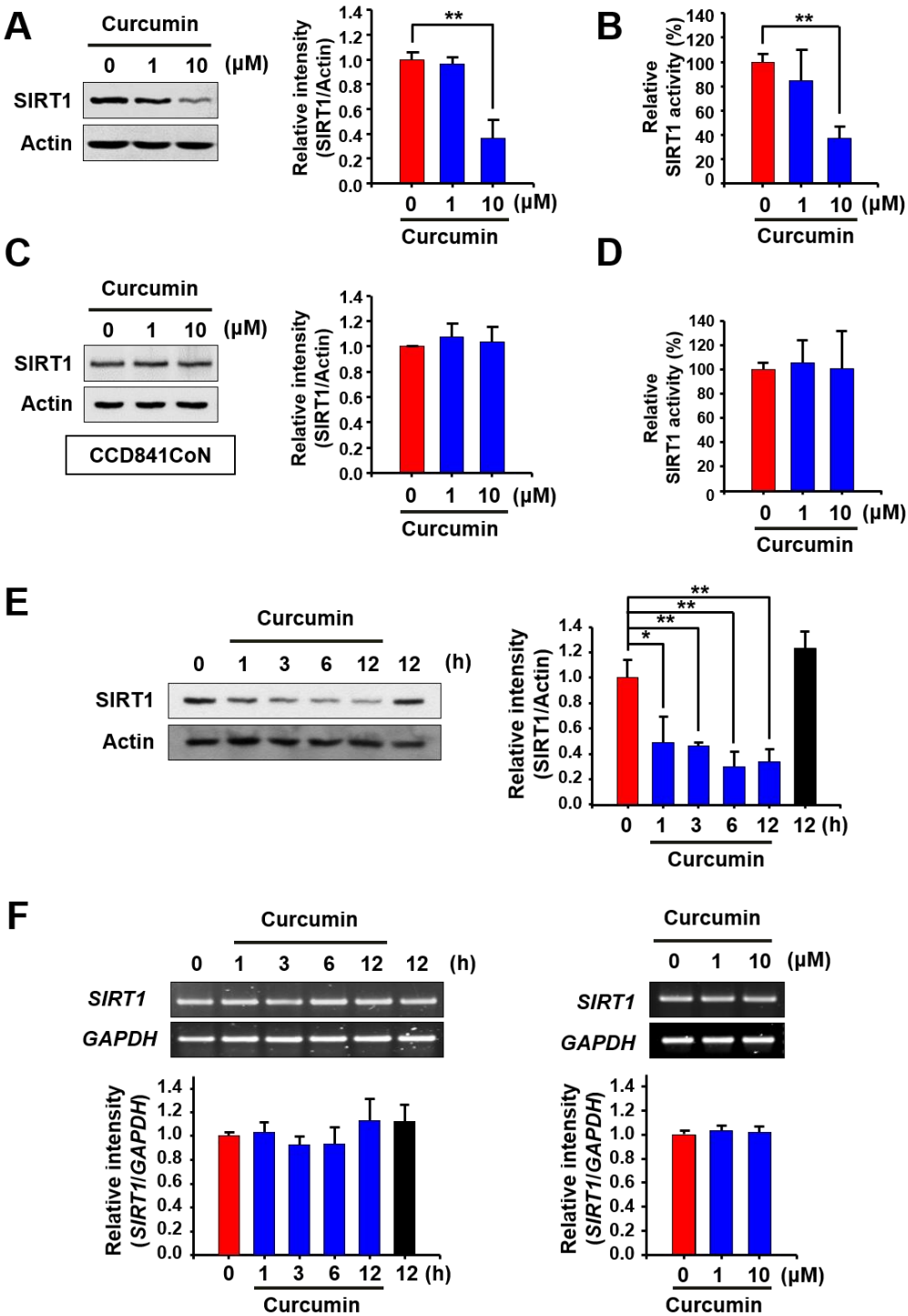
Cysteine 67 modification by curcumin induces conformational changes in SIRT1 that would affect its protein stability

We next examined the involvement of cysteine 67 in curcumin-induced destabilization of SIRT1 protein. When the protein level of SIRT1 was measured, HCT-116 cells transfected with SIRT1-WT showed decreased stability of SIRT1 upon curcumin treatment. However, the protein level of SIRT1 in HCT-116 cells harbouring mutant SIRT1 (SIRT1-C67A) was not diminished in the presence of curcumin (**Fig. 3-5A**). Consistent with this finding, curcumin barely induced the ubiquitination of SIRT1 in HCT-116 cells expressing SIRT1-C67A (**Fig. 3-5B**). The above results indicate that binding of curcumin to cysteine 67 of SIRT1 is important in the ubiquitination-dependent degradation of SIRT1. In parallel with these findings, anchorage-independent growth of cells expressing cysteine 67 mutant SIRT1 was affected by curcumin to a lesser extent than cells expressing SIRT1-WT (**Fig 3-5C**). Similar result was also seen in the wound healing assay (**Fig. 3-5D).**

Interaction between SIRT1 and c-Jun N-terminal kinase (JNK) may be disrupted by curcumin-mediated cysteine 67 modification of SIRT1

It has been suggested that abnormal stabilization of SIRT1 protein in human colon cancer (HCT-116) cells is associated with its increased phosphorylation at serine 27 residue, and JNK appears to be a putative kinase (26). Indeed, SP600125, a selective inhibitor of JNK, reduced the levels of phospho- and total SIRT1 **(Fig. 4-2B**), but not the mRNA level **(Fig. 4-3A**), suggesting that JNK posttranslationally regulates SIRT1. In addition, SP600125 provoked ubiquitination-dependent degradation of SIRT1 **(Fig. 4-3B**), resulting in its decreased stability **(Fig. 4-3C**). When mutant SIRT1 (SIRT1-C67A) was resistant to curcumin, thereby preserving the protein stability, the phosphorylation level of SIRT1 at serine 27 residue was also sustained (**Fig. 3-6A**). Considering that the cells expressing serine to aspartic acid substitution mutant (SIRT1- S27D), which mimics phosphorylation status, had increased protein level of SIRT1 compared to wild-type SIRT1 **(Fig. 4-1D**), the observation shown in **Fig. 3-6A** was not due to persistent total level of SIRT1. Based on above results, we speculated that binding of curcumin to SIRT1 may disrupt the interaction between JNK and SIRT1, thereby causing the blockade of the phosphorylation-dependent stabilization of SIRT1. The results indicated that curcumin weakened the binding of phosphorylated JNK to SIRT1 in HCT-116 cells harbouring SIRT1-WT, whereas curcumin barely inhibited the interaction

between phosphorylated JNK and SIRT1 in HCT-116 cells expressing SIRT1-C67A (**Fig. 3-6B**). These findings suggest that cysteine 67 modification of SIRT1 by curcumin is a prerequisite for dephosphorylation-mediated degradation of SIRT1.


A
Curcumin
0 1 10 (μM)
SIRT1
Actin
Relative intensity (SIRT1/Actin)
**
0 1 10 (μM)
Curcumin
B
Relative SIRT1 activity (%)
**
0 1 10 (μM)
Curcumin
C
Curcumin
0 1 10 (μM)
SIRT1
Actin
CCD841CoN
Relative intensity (SIRT1/Actin)
0 1 10 (μM)
Curcumin
D
Relative SIRT1 activity (%)
0 1 10 (μM)
Curcumin
E
Curcumin
0 1 3 6 12 12 (h)
SIRT1
Actin
Relative intensity (SIRT1/Actin)
**
**
**
*
0 1 3 6 12 12 (h)
Curcumin
F
Curcumin
0 1 3 6 12 12 (h)
SIRT1
GAPDH
Relative intensity (SIRT1/GAPDH)
0 1 3 6 12 12 (h)
Curcumin
Curcumin
0 1 10 (μM)
SIRT1
GAPDH
Relative intensity (SIRT1/GAPDH)
0 1 10 (μM)
Curcumin

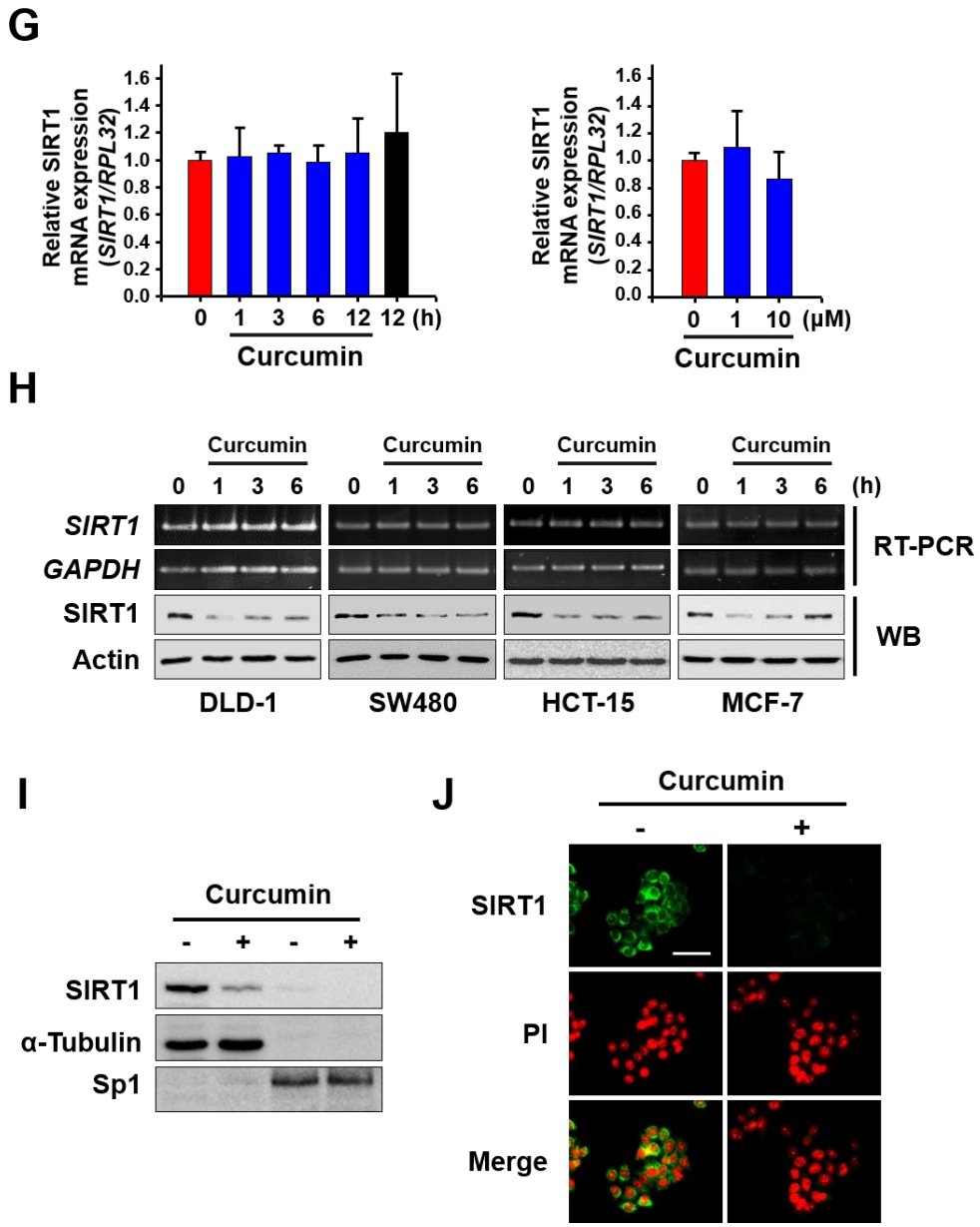


Fig. 3-1. Effects of curcumin on the expression and activity of SIRT1 in cultured human colon cancer HCT-116 cells. (A) HCT-116 cells were treated with two different concentrations (1 or 10 μM) of curcumin for 3 h. The protein level of SIRT1 was measured

by Western blot analysis. The results are shown as the mean ± S.D. of three independent experiments, $^{**}p < 0.01$. (B) Following treatment of HCT-116 cells with curcumin (1 or 10 μM) for 3 h, SIRT1 deacetylase activity was measured by using the fluorometric assay kit as described in Materials and Methods. The data are presented as the mean ± S.D. of three independent experiments, $^{**}p < 0.01$. (C) CCD841CoN cells were treated with 1 or 10 μM of curcumin for 3 h. The protein level of SIRT1 was measured by Western blot analysis. (D) Following treatment of CCD841CoN cells with curcumin (1 or 10 μM) for 3 h, SIRT1 deacetylase activity was measured by using the fluorometric assay kit. (E) HCT-116 cells were treated with curcumin (10 μM) for the indicated durations. The protein level of SIRT1 was measured by immunoblot analysis. (F, G) HCT-116 cells were treated with curcumin (10 μM) for the indicated time periods or with 1 or 10 μM for 3 h. The mRNA levels of SIRT1 were measured by RT-PCR (F) and real-time PCR (G) analyses using GAPDH and RPL32, respectively as an equal loading control for normalization. (H) DLD-1, SW480, HCT-15 and MCF-7 cells were treated with curcumin (10 μM) for the indicated durations. The protein and mRNA levels of SIRT1 were measured by Western blot and RT-PCR analyses, respectively. (I) Following exposure of HCT-116 cells to curcumin (10 μM) for 3 h, both cytosolic and nuclear extracts were prepared and subjected to immunoblot analysis. α-Tubulin and Sp1 were used as cytosolic and nuclear markers, respectively. (J) Immunocytochemical analysis was

performed using anti-SIRT1 antibody after the treatment of HCT-116 cells with curcumin (10 μM) for 3 h. Nuclei were identified by propidium iodide (PI) staining. The scale bar represents 100 μm.

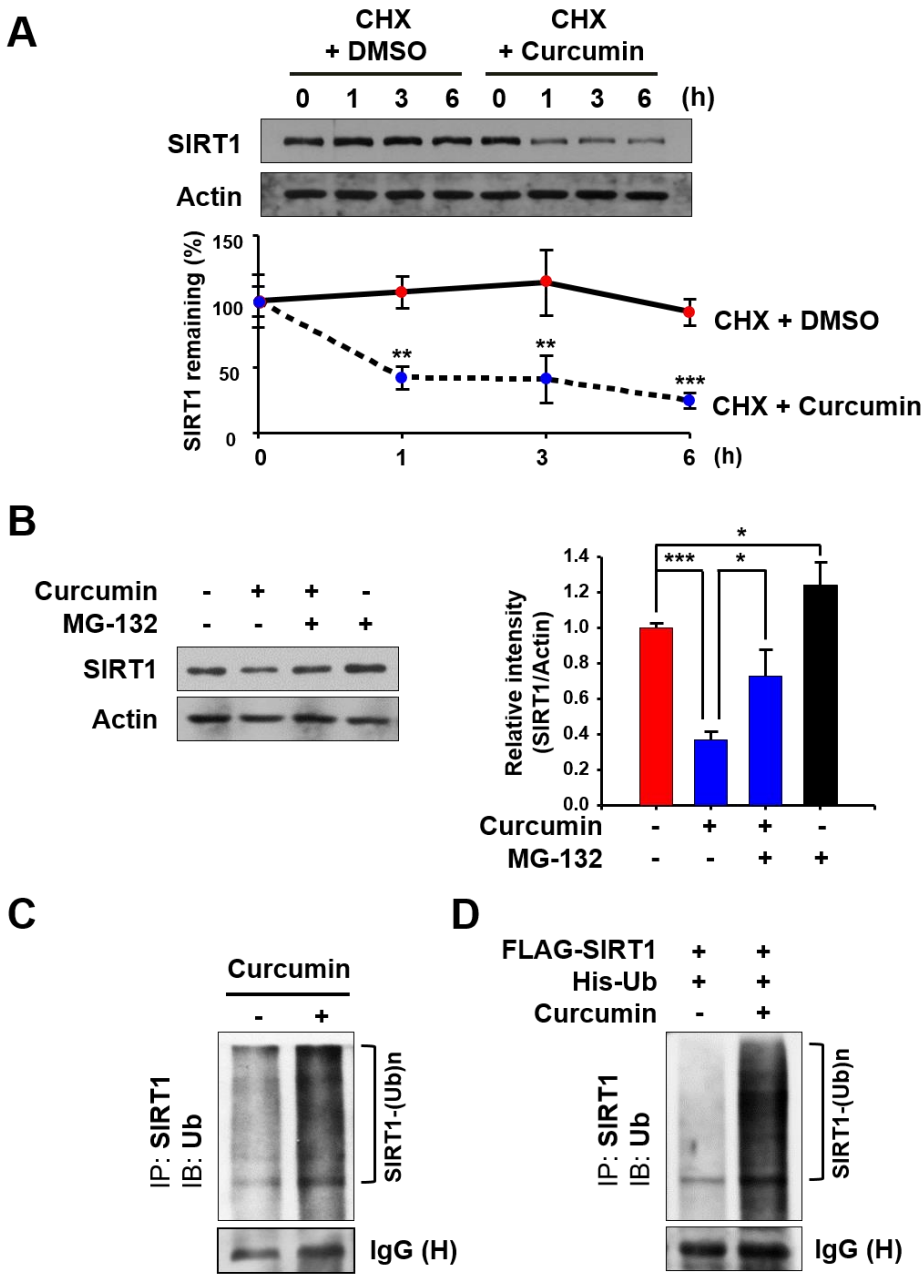
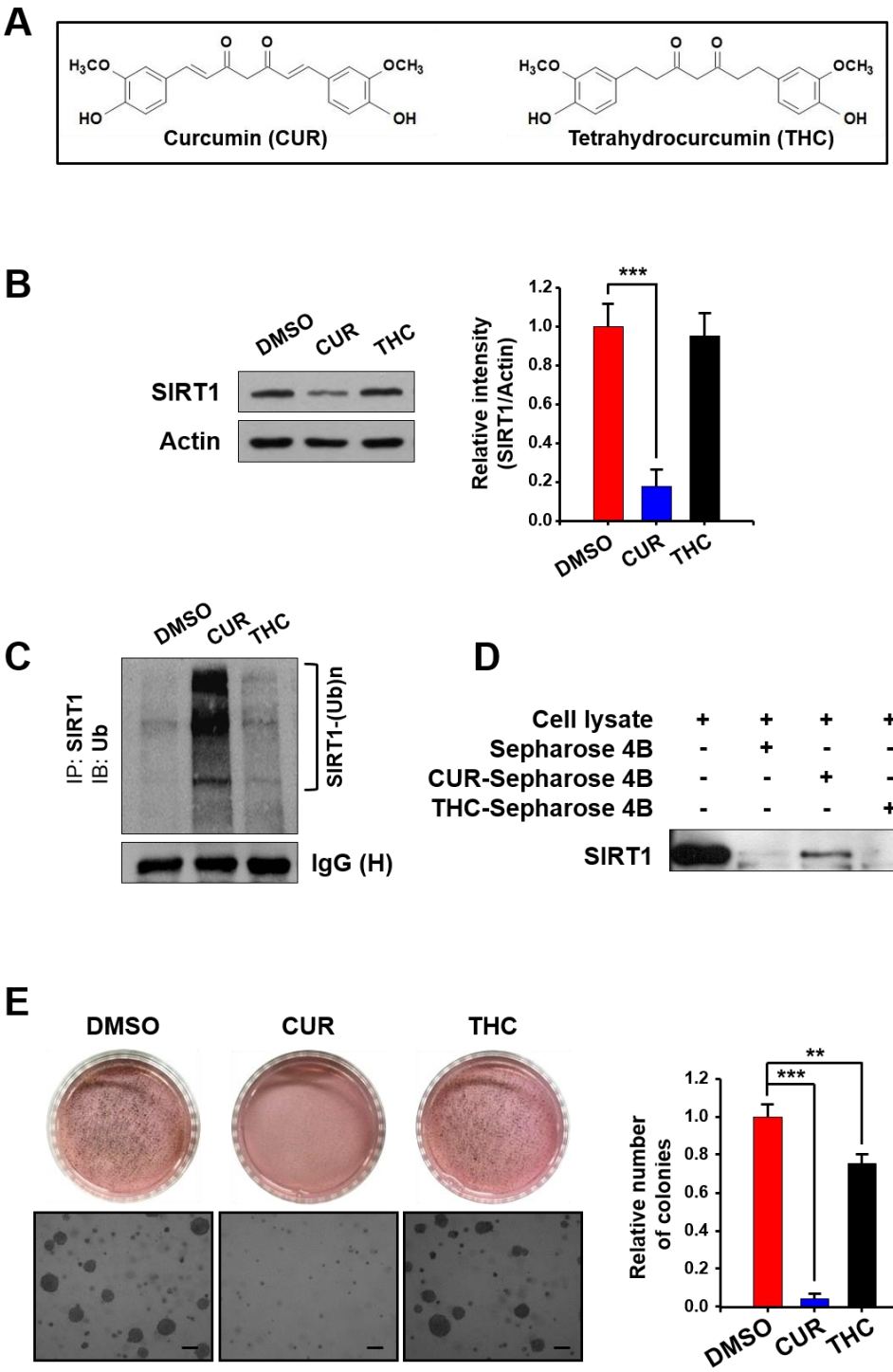
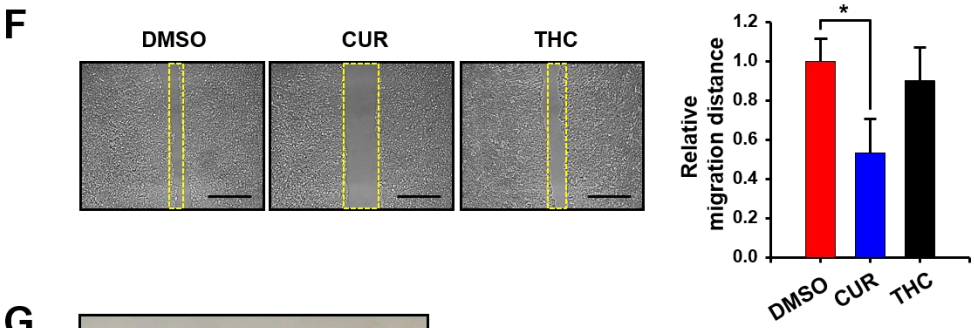
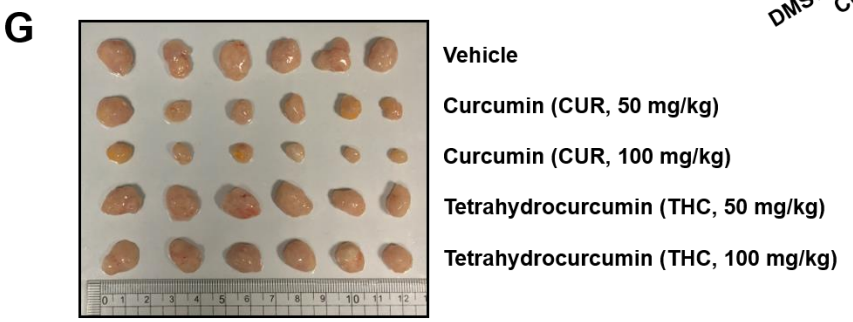


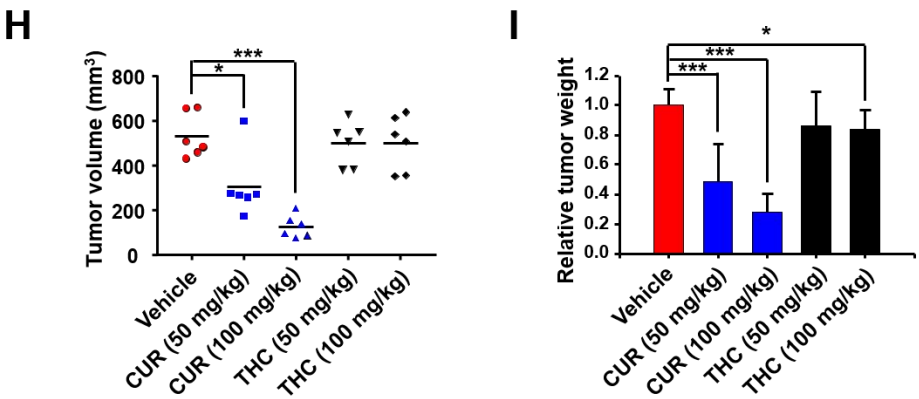
Fig. 3-2. Stimulation of ubiquitin-dependent proteasomal degradation of SIRT1 by curcumin. (A) HCT-116 cells were treated with cycloheximide (CHX; 71.07 μM) or vehicle for indicated periods in the absence or presence of curcumin (10 μM). The relative protein

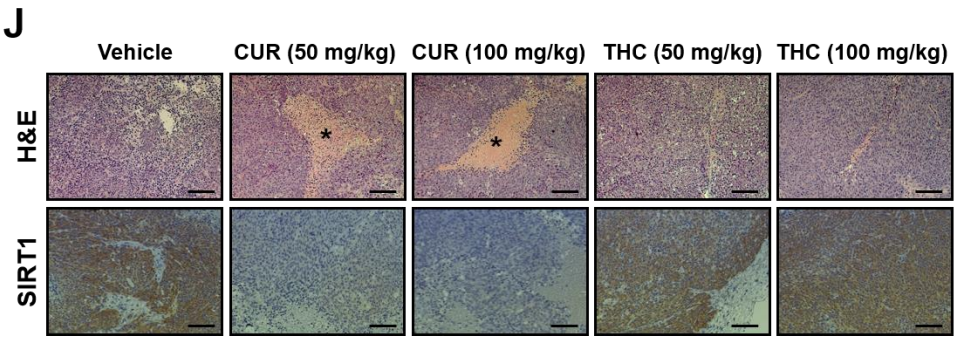
levels of SIRT1 remaining at different time points were assessed. The results are shown as the mean ± S.D. of three independent experiments, $^{**}p < 0.01$, $^{***}p < 0.001$. (B) HCT-116 cells were exposed to curcumin (10 μM) alone or in combination with a proteasome inhibitor MG-132 (20 μM), and the protein level of SIRT1 was examined by Western blot analysis. The values are presented as the mean ± S.D. of three independent experiments, $^{*}p < 0.05$, $^{***}p < 0.001$. (C) Following treatment of HCT-116 cells with MG-132 (20 μM) and curcumin (10 μM) for 3 h, the ubiquitination of endogenous SIRT1 was measured by immunoprecipitation. (D) HCT-116 cells were co-transfected with FLAG-SIRT1 and His-ubiquitin for 48 h, followed by MG-132 (20 μM) and curcumin (10 μM) treatment for 3 h. The ubiquitination of exogenous SIRT1 was measured by immunoprecipitation.


A
Curcumin (CUR)
Tetrahydrocurcumin (THC)
B
DMSO
CUR
THC
SIRT1
Actin
Relative intensity (SIRT1/Actin)
***
C
IP: SIRT1
IB: Ub
SIRT1-(Ub)n
IgG (H)
D
Cell lysate
Sepharose 4B
CUR-Sepharose 4B
THC-Sepharose 4B
SIRT1
E
DMSO
CUR
THC
Relative number of colonies
**
***


F
DMSO
CUR
THC
Relative migration distance
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
DMSO
CUR
THC


G
Vehicle
Curcumin (CUR, 50 mg/kg)
Curcumin (CUR, 100 mg/kg)
Tetrahydrocurcumin (THC, 50 mg/kg)
Tetrahydrocurcumin (THC, 100 mg/kg)


H
Tumor volume (mm3)
800
600
400
200
0
*
***
Vehicle
CUR (50 mg/kg)
CUR (100 mg/kg)
THC (50 mg/kg)
THC (100 mg/kg)
I
Relative tumor weight
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
***
***
Vehicle
CUR (50 mg/kg)
CUR (100 mg/kg)
THC (50 mg/kg)
THC (100 mg/kg)


J
Vehicle
CUR (50 mg/kg)
CUR (100 mg/kg)
THC (50 mg/kg)
THC (100 mg/kg)
H&E
SIRT1
*
*

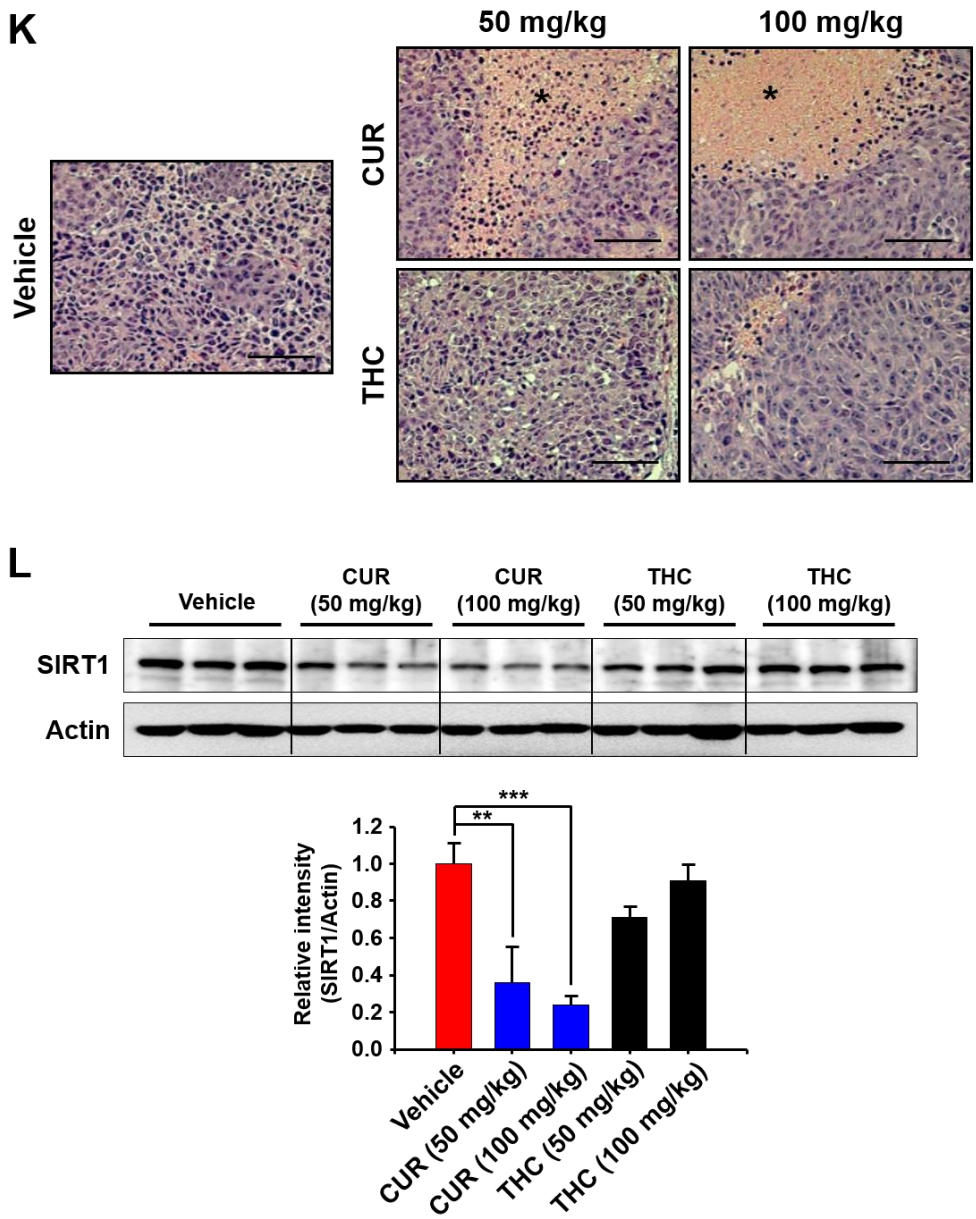
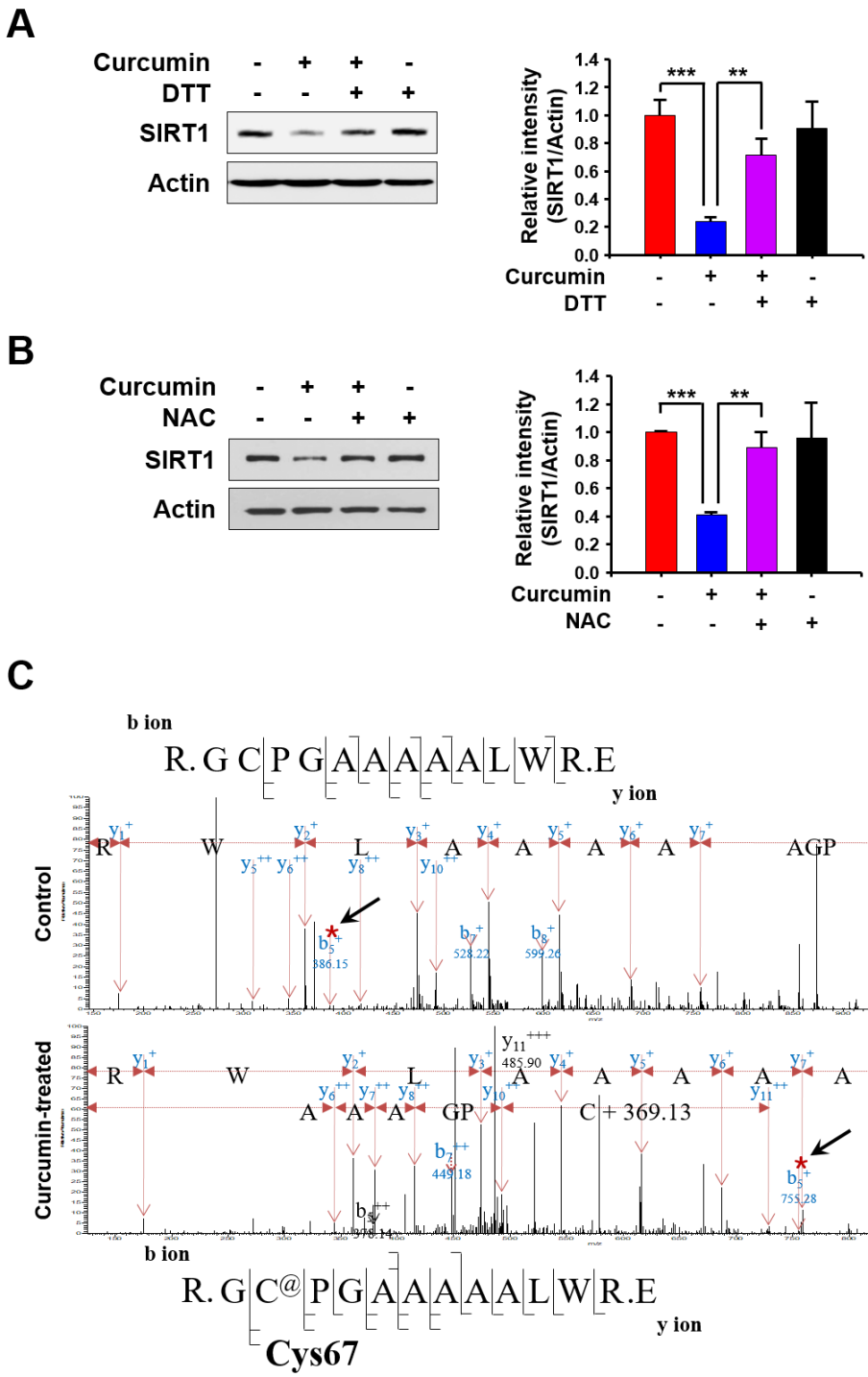


Fig. 3-3. Comparison of the effects of curcumin and tetrahydrocurcumin on the stability of SIRT1, colon cancer cell transformation and the growth of xenograft tumors. (A) Chemical structures of curcumin and its non-electrophilic analogue, tetrahydrocurcumin.

Unlike curcumin, tetrahydrocurcumin lacks the conjugated bonds in the central seven-carbon chain. (B) Following treatment of HCT-116 cells with curcumin (10 μM) or tetrahydrocurcumin (10 μM), the protein level of SIRT1 was examined by Western blot analysis. The data are presented as the mean ± S.D. of three independent experiments, ***$p < 0.001$. (C) The ubiquitination of SIRT1 was measured by immunoprecipitation after treatment of HCT-116 cells with curcumin (10 μM) or the same concentration of tetrahydrocurcumin. (D) Whole-cell lysates from HCT-116 cells were incubated overnight at 4 °C with Sepharose 4B beads conjugated to curcumin or tetrahydrocurcumin and were then pulled down by centrifugation. Covalent interaction between SIRT1 and curcumin or tetrahydrocurcumin was assessed by immunoblot analysis using an antibody against SIRT1. (E) For the anchorage-independent growth assay, HCT-116 cells were grown in soft agar for 14 days treated with 10 μM each of curcumin or tetrahydrocurcumin every other day and were then stained with 0.05% crystal violet. The number of colonies larger than 100 μm in diameter was counted. Each scale bar represents 200 μm. The results are shown as the mean ± S.D. of three independent experiments performed, **$p < 0.01$, ***$p < 0.001$. (F) HCT-116 cells were exposed to curcumin (10 μM) or tetrahydrocurcumin (10 μM) for 48 h, and the cell migration was assessed. Each scale bar represents 500 μm. The values are resented as the mean ± S.D. of three independent experiments, *$p < 0.05$. (G) Photograph of the xenograft tumors from

the mice treated with curcumin or tetrahydrocurcumin at the time of excision. The treatment of mice and other experimental details are described in Materiald and Methods. (H) The tumor volume of the mice at the end of the experiment. The results are shown as the mean ± S.D. of six xenografts for each group, *$p < 0.05$, ***$p < 0.001$. (I) The tumor weight of the mice at the end of the experiment. The data are presented as the mean ± S.D. of six samples for each group, *$p < 0.05$, ***$p < 0.001$. (J) Histological morphology was assessed by H&E staining (upper panels), and the protein level of SIRT1 was examined by immunohistochemical staining (lower panels) in sections from resected xenograft (magnification, × 10). The asterisk indicates the necrotic area. Each scale bar represents 200 μm. (K) Higher magnification images corresponding to the **Fig. 3J**. The asterisk indicates the necrotic area. Each scale bar represents 300 μm. (L) The protein expression of SIRT1 in the xenograft tumors from the mice treated with curcumin or tetrahydrocurcumin. The values are presented as the mean ± S.D. of six samples, **$p < 0.01$, ***$p < 0.001$.


A
Curcumin - + + -
DTT - - + +
SIRT1
Actin
Relative intensity (SIRT1/Actin)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
***
**
Curcumin - + + -
DTT - - + +
B
Curcumin - + + -
NAC - - + +
SIRT1
Actin
Relative intensity (SIRT1/Actin)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
***
**
Curcumin - + + -
NAC - - + +
C
b ion
R.G C P G A A A A A L W R.E
y ion
Control
R W L A A A A AGP
b5+ 386.15
b7+ 528.22
b8+ 599.26
b11+ 969.46
Curcumin-treated
R W L A A A A A A G
A A A GP C + 369.13
y11+++ 485.90
b7++ 449.18
b8+ 755.28
b ion
R.G C@ P G A A A A A L W R.E
y ion
Cys67

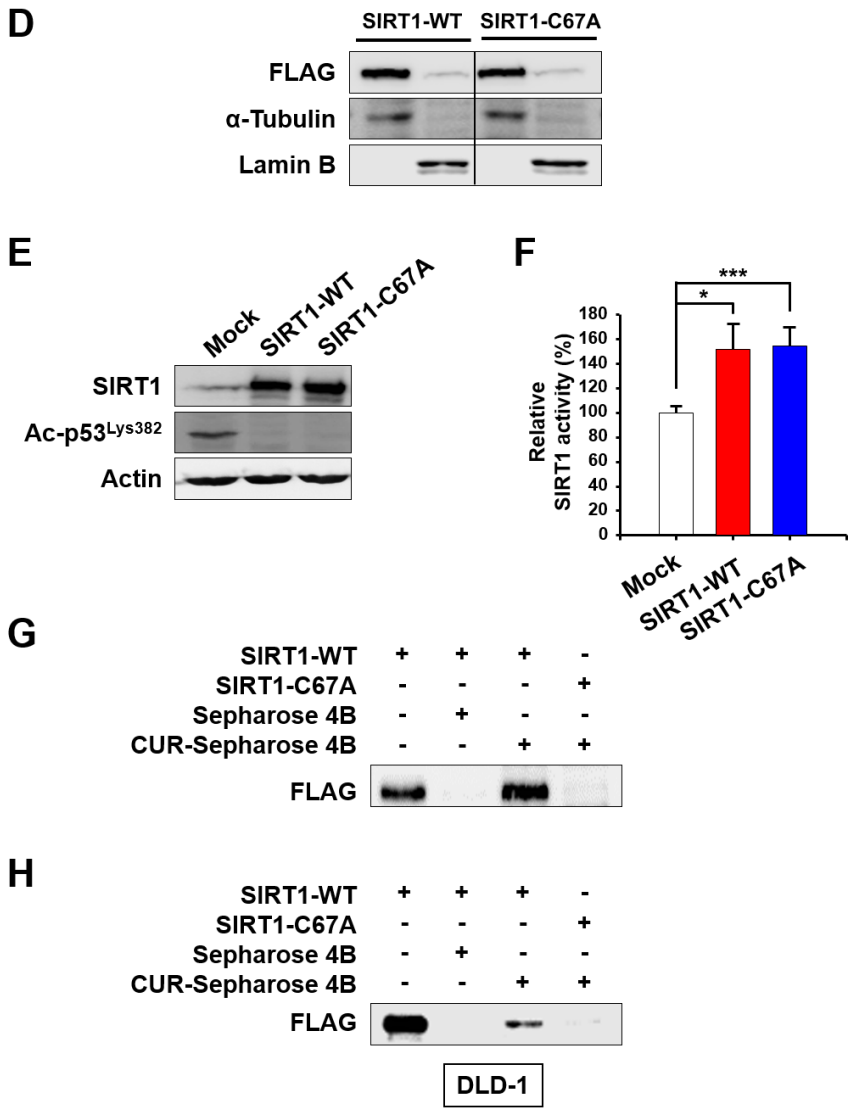


Fig. 3-4. The cysteine 67 residue of SIRT1 as a binding site of curcumin. (A, B) HCT-116 cells were exposed to curcumin (10 μM) in the presence or absence of a thiol reducing agent DTT (500 μM) (A) or NAC (10 mM) (B). The results are shown as the mean ± S.D. of three independent experiments, $^{**}p < 0.01$, $^{***}p < 0.001$. (C) Recombinant human SIRT1 (5 μg) was mixed with curcumin (10 μM) or vehicle (DMSO) for 1 h at room temperature and

subjected to nano-LC-ESI-MS/MS. Data were analyzed using BioWorksBrowser™ as described in Materials and Methods. (D) HCT-116 cells were transiently transfected with FLAG-tagged wild-type (SIRT1-WT) or mutant-SIRT1 (SIRT1-C67A). Both cytosolic and nuclear extracts were then prepared and subjected to Western blot analysis. α-Tubulin and Lamin B were used as cytosolic and nuclear markers, respectively. (E) HCT-116 cells were transiently transfected with wild-type (SIRT1-WT) or mutant-SIRT1 (SIRT1-C67A), and the protein level of Ac-p53^{Lys382} was determined by immunoblot analysis. (F) HCT-116 cells were transiently transfected with wild-type (SIRT1-WT) or mutant-SIRT1 (SIRT1-C67A), and the deacetylase activity of SIRT1 was measured by using the fluorometric assay kit. (G) HCT-116 cells were transiently transfected with the FLAG-tagged plasmids expressing wild-type SIRT1 (SIRT1-WT) or mutant-SIRT1 (SIRT1-C67A). Whole-cell lysates were incubated with curcumin-Sepharose 4B beads. The SIRT1 bound to the curcumin-Sepharose 4B beads was pulled down by centrifugation and analyzed by Western blot analysis using anti-FLAG antibody. (H) DLD-1 cells were transiently transfected with the FLAG-tagged plasmids expressing wild-type SIRT1 (SIRT1-WT) or mutant-SIRT1 (SIRT1-C67A). Following incubation of whole-cell lysates from DLD-1 cells with curcumin-Sepharose 4B beads, the SIRT1 bound to the curcumin-Sepharose 4B beads was pulled down by centrifugation and analyzed by immunoblot analysis using anti-FLAG antibody.

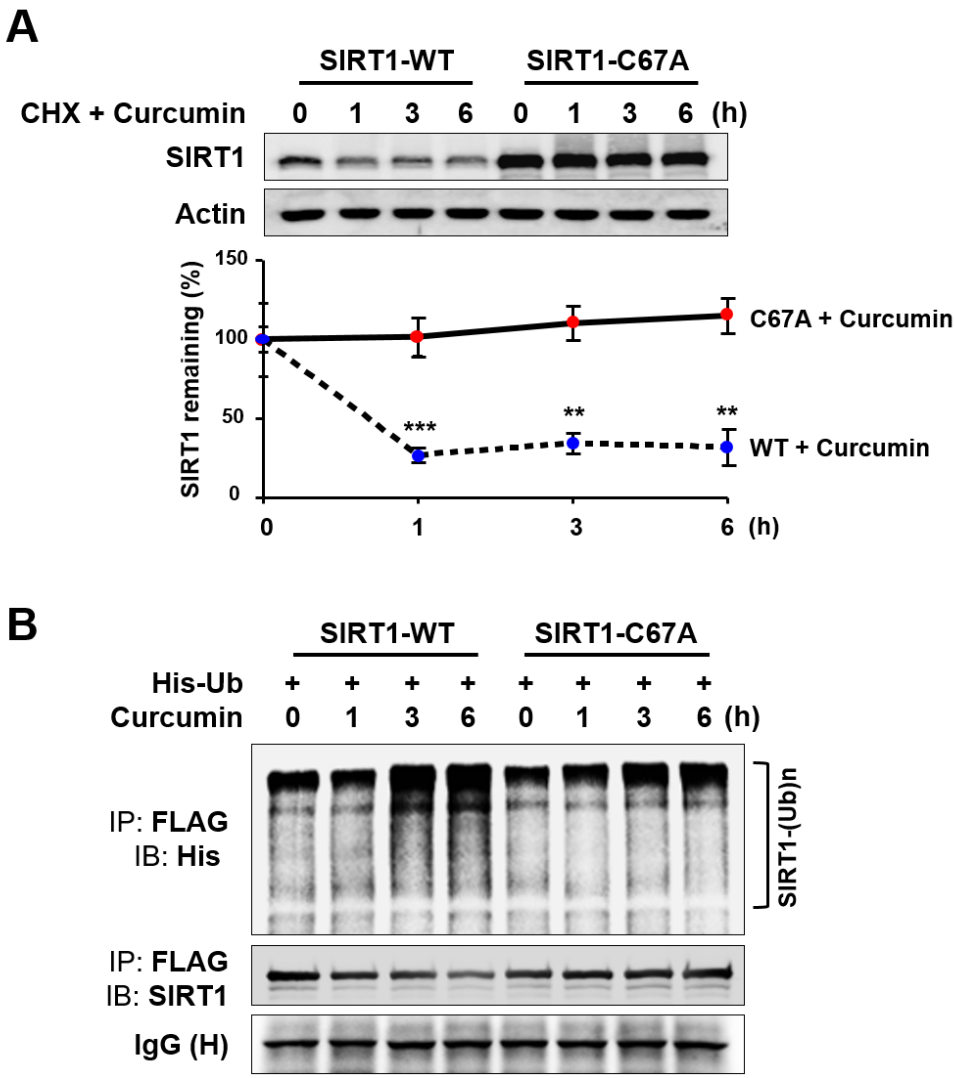
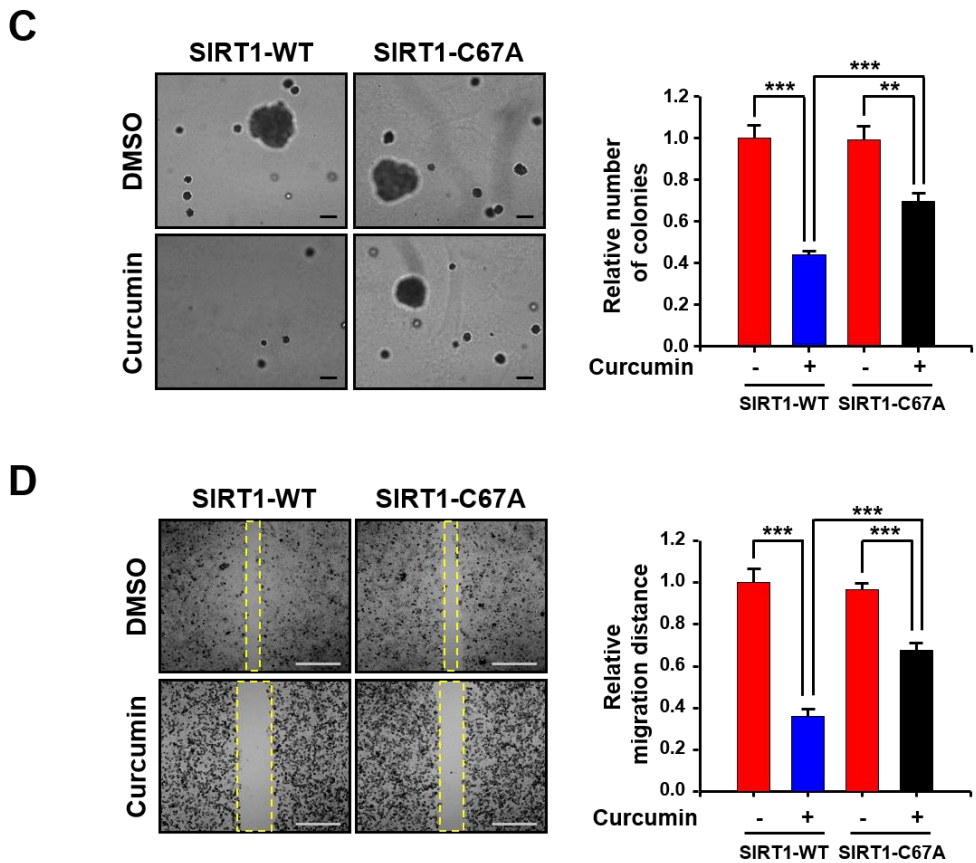


Fig. 3-5. Suppression of curcumin-induced ubiquitin-dependent proteasomal degradation of SIRT1 and anchorage-independent growth in HCT-116 cells by mutation of SIRT1 cysteine 67. (A) HCT-116 cells were treated with curcumin (10 μM) and cycloheximide (CHX; 71.07 μM) for indicated time periods following transfection with wild-type (SIRT1-WT) or mutant-SIRT1 (SIRT1-C67A), and the protein level of SIRT1 remaining

at each time point was determined by immunoblot analysis. The results are shown as the mean ± S.D. of three independent experiments, $^{**}p < 0.01$, $^{***}p < 0.001$. (B) HCT-116 cells were transiently transfected with the His-ubiquitin plasmids and FLAG-tagged wild-type SIRT1 (SIRT1-WT) or mutant-SIRT1 (SIRT1-C67A). Following treatment of HCT-116 cells with MG-132 (20 μM) and curcumin (10 μM) for the indicated durations, the ubiquitination of SIRT1 was measured by immunoprecipitation.



(C) HCT-116 cells were transiently transfected with wild-type or mutant SIRT1. Cells were then treated with curcumin (10 μM) for the anchorage-independent growth assay as described in Materials and Methods. Each scale bar represents 200 μm. The data are presented as the mean ± S.D. of three independent experiments, $^{**}p < 0.01$, $^{***}p < 0.001$. (D) HCT-116 cells were transiently transfected with wild-type (SIRT1-WT) or mutant-SIRT1 (SIRT1-C67A). Cells were then treated with curcumin (10 μM) or vehicle for the wound healing assay as described in Materials and Methods. Each scale bar represents 500 μm. The values are presented as the mean ± S.D. of triplicates, $^{***}p < 0.001$.

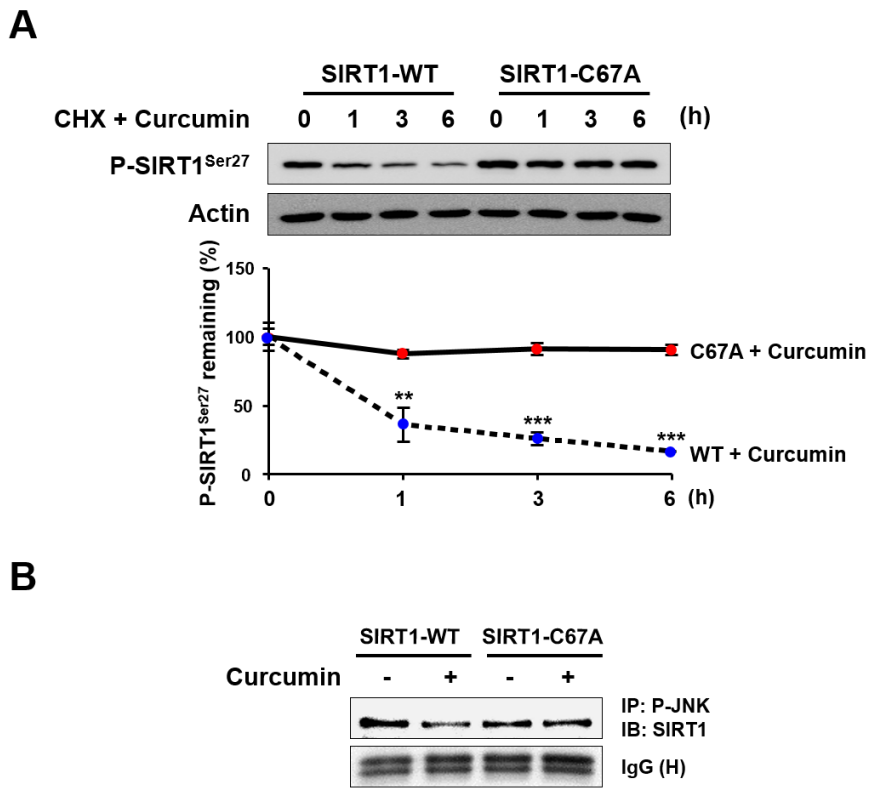


Fig. 3-6. Curcumin-induced covalent modification of the cysteine 67 residue of SIRT1 inhibits its phosphorylation by JNK. (A) HCT-116 cells were treated with curcumin (10 μM) and cycloheximide (CHX; 71.07 μM) for the indicated durations following transfection with wild-type (SIRT1-WT) or mutant-SIRT1 (SIRT1-C67A), and the protein level of phospho-SIRT1^{Ser27} remaining at each time point was determined by immunoblot analysis. The data are presented as the mean ± S.D. of three independent experiments, $^{**}p < 0.01$, $^{***}p < 0.001$. (B) HCT-116 cells were transfected with wild-type (SIRT1-WT) or mutant-SIRT1 (SIRT1-C67A) for 48 h. Following treatment of cells with curcumin (10 μM) for 3 h, the interaction between phospho-JNK and SIRT1 was assessed by immunoprecipitation.

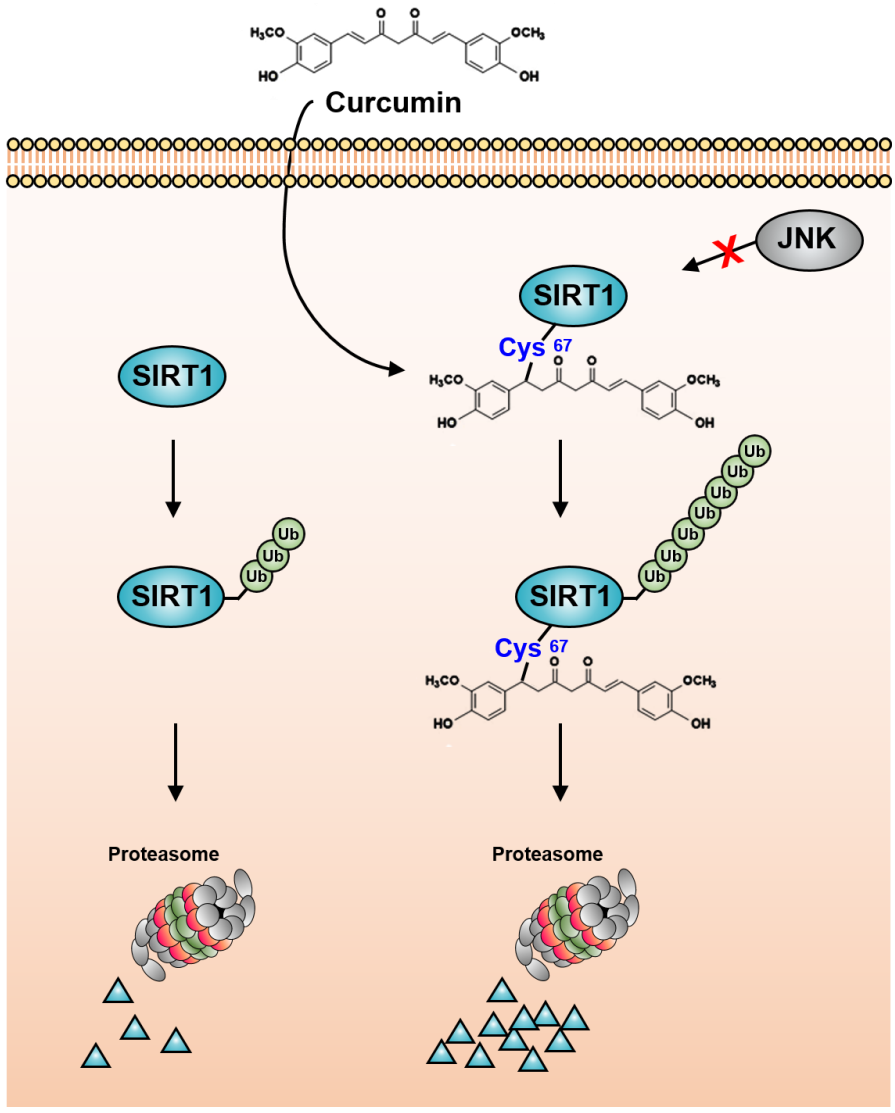


Fig. 3-7. A proposed mechanism underlying curcumin-induced destabilization of oncogenic SIRT1. Curcumin bearing the α,β-unsaturated carbonyl moiety can directly bind to SIRT1 protein, preferentially at the cysteine 67 residue. The resulting conformational alteration of the SIRT1 structure is speculated to facilitate its degradation through the ubiquitin-proteasome system.

5. Discussion

Meta-analysis of gastrointestinal cancers revealed that the expression of SIRT1 was not correlated with worse overall survival in CRC (27). However, another meta-analysis showed that the expression of SIRT1 is associated with the poor prognosis in CRC patients (28). While there is some discrepancy regarding the role of SIRT1 in tumor progression, recent studies have revealed that SIRT1 is abnormally overexpressed in CRC tissues (11). Notably, the elevated levels of SIRT1 are significantly associated with not only tumor invasion and lymph node metastasis (29-31), but higher grades of malignancy and mutations of *KRAS* and *BRAF* (32), suggesting the prognostic utility of SIRT1.

In a phase IIa cancer prevention trial with participants with rectal aberrant crypt foci (ACF), daily administration (*p.o.*) of 4 g curcumin for 30 days significantly reduced ACF formation after 30 days of daily oral administration, resulting in 7.3 ± 8.1 ng/ml (approximately 20 nM) venous plasma concentration of curcumin (33). In a phase I clinical study, a daily oral consumption of 3.6 g curcumin for up to 4 months resulted in a plasma concentration of 11.1 nM and also decreased serum levels of prostaglandin E_2 in the CRC patients (34). In another phase I clinical trial, oral consumption of 4-8 g of curcumin for 3 months resulted in a peak plasma level of 0.51-1.77 μM in volunteers with high risk conditions including intestinal metaplasia (35). Such low bioavailability of curcumin is attributable to its

poor absorption from the gut. Curcumin in cultured cells also undergoes rapid metabolism. Thus, only less than 2% of curcumin was detected after 3 h incubation in human colon cancer (Caco-2) cells (36).

It has been reported that curcumin can activate or upregulate SIRT1 in some cases (37-40). Curcumin has been reported to exert preventive effects against pathogenesis of several disorders by activating SIRT1 in cells or tissues of noncancerous origin. When normal isolated rat hearts were exposed to ischemia reperfusion (IR) injury, the expression of SIRT1 decreased. However, curcumin pretreatment protects the SIRT1 from IR-induced loss in isolated hearts (37). In another study, the level of SIRT1 in cultured neurons isolated from healthy *Sprague–Dawley* rats was substantially decreased by $A\beta_{25–35}$, a neurotoxic peptide accumulated in the brain of patients with Alzheimer's disease. Curcumin pretreatment attenuated the loss of cellular accumulation of SIRT1, resulting in enhanced neuronal survival against $A\beta_{25–35}$-induced toxicity (38). Curcumin induced upregulation of SIRT1 in vascular smooth muscle (VSMC) cells, which was speculated to account for the anti-aging effect of curcumin in these cells (40). The above findings suggest involvement of SIRT1 activation in health benefits of curcumin. Interestingly, even in malignant cells, activation of SIRT1 contributes to anticarcinogenic effect of curcumin. For instance, curcumin induces ATM-mediated apoptotic signaling through activation of SIRT1 in head and neck squamous cell carcinomas (FaDu and

Cal27) cells (39).

In contrast to the aforementioned findings, we found that the protein levels of SIRT1 declined upon treatment with curcumin in 4 different human CRC cell lines (HCT-116, DLD-1, HCT-15 and SW480). Interestingly, the SIRT1 protein level in normal colonic epithelial cells was not affected by curcumin. Besides differences in amounts and subcellular localization of SIRT1 between normal and cancer cells (10, 41), there might be some distinguishable factors, such as intracellular redox environment surrounding the SIRT1 protein that may confer the differential effects of curcumin on SIRT1 protein level or activity.

Increased protein expression of SIRT1 in CRC and hepatocellular carcinoma is not attributable to elevated mRNA expression of SIRT1, suggesting that overexpression of SIRT1 is correlated with its increased protein stability (42, 43). Nonetheless, much less is known about regulation of SIRT1 protein stability. A very few studies have suggested that deubiquitination (44) and phosphorylation of SIRT1 at serine 27 and 47 are associated with the enhanced SIRT1 protein stability (26, 45). Substantial differences between curcumin and its non-electrophilic analogue tetrahydrocurcumin in their effects on SIRT1 accumulation in HCT-116 cells lead us to speculate that curcumin-induced destabilization of SIRT1 protein is attributable to its electrophilic nature. We note that curcumin is more effective than tetrahydrocurcumin in reducing tumor growth in a murine xenograft model, highlighting the

unique structural feature of curcumin essential for its biological activities.

Curcumin has two electrophilic α,β-unsaturated carbonyl groups, and hence can act as a Michael reaction acceptor. There are several reports suggesting that curcumin can directly modify cysteine thiols of cellular proteins by Michael addition, thereby modulating their functions (21, 23, 46). SIRT1 contains 19 cysteine residues, and some of which are susceptible to redox modification. It has been reported that the cysteine 67 residue of SIRT1 is directly modified by *S*-nitrosoglutathione formed from nitric oxide and glutathione, which attenuates activation of SIRT1 by resveratrol (47). In addition, cysteine 371 and 374 residues present in the catalytic domain of SIRT1 are targeted for reduction by redox factor-1 (Ref-1). Ref-1, as a cellular reductant, stimulates SIRT1 activity by keeping SIRT1 in the reduced form (48). It has also been shown that the cysteine 482 residue of SIRT1 is covalently modified by 4-hydroxy-2-nonenal (4-HNE), a reactive hydroxyalkenal produced by lipid peroxidation. Carbonylation of SIRT1 by 4-HNE results in inhibition of deacetylase activity and protein expression of SIRT1 (49).

In the current study, nano-LC-ESI-MS/MS analysis revealed that curcumin directly binds to the cysteine 67 residue located in the N-terminal domain of SIRT1 which functions as a linker in interacting with its substrate proteins (50, 51). Our results show that cysteine 67 to alanine substitution renders HCT-116 colon cancer cells insensitive to curcumin-mediated

inhibition of their clonogenicity and proteasomal degradation of SIRT1. Thus, it is plausible that covalent thiol modification at the cysteine 67 residue contributes to destabilization of SIRT1 by curcumin. As replacement of the cysteine 67 residue by alanine hampers direct interaction of SIRT1 with curcumin, it is likely that cysteine 67 is a bona fide binding site of curcumin. We speculate that direct binding of curcumin to cysteine 67 might cause conformational changes of SIRT1, leading to the protrusion of lysine residues that can be ubiquitinated. This allows E3 ubiquitin ligases to have better access to the SIRT1. Unfortunately, the crystallographic structure of the full-length human SIRT1 has not been solved yet, so the alteration in the structure of SIRT1 complexed with curcumin cannot be verified at this moment.

In conclusion, curcumin that contains the electrophilic α,β-unsaturated carbonyl moiety can directly bind to SIRT1 protein, preferentially at the cysteine 67 residue. The resulting structural modification facilitates the ubiquitin-dependent proteasomal degradation of oncogenic SIRT1 overexpressed in colon cancer cells (**Fig. 3-7**). This may account for suppression of CRC progression by curcumin.

6. References

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Chapter IV

Curcumin induces proteasomal degradation of SIRT1 by inhibiting phosphorylation of its serine 27 residue in human colon cancer HCT-116 cells

1. Abstract

SIRT1, an NAD^+-dependent histone/protein deacetylase, has multifaceted functions in various biological events such as inflammation, apoptosis, aging and energy metabolism. The role of SIRT1 in carcinogenesis, however, is still under debate. Recent studies have indicated that aberrant overexpression of SIRT1 is correlated with metastasis and poor prognosis in several types of cancer, suggesting that it may act as a tumor promoter. SIRT1 is known to be stabilized through its phosphorylation at the serine 27 residue. In support of this notion, phosphorylation-defective mutant SIRT1 (SIRT1-S27A) exhibited lower protein stability compared to that of wild-type SIRT1. Curcumin (diferuloymethane), a substance present in the spice turmeric (*Curcuma longa* L., Zingiberaceae) has been known to possess the beneficial effects on pathological conditions such as inflammation and cancer. In the present study, I found that curcumin inhibited phosphorylation of SIRT1 at the serine 27 residue coupled with its proteasomal degradation in human colon cancer (HCT-116) cells. Serine 27 to aspartic acid substitution mutant SIRT1 (SIRT1-S27D) mimicking the phosphorylation status showed a prolonged half-life upon curcumin treatment compared to that of wild-type SIRT1. Pharmacologic inhibition of c-Jun N-terminal kinase (JNK) abrogated SIRT1 phosphorylation at the serine 27, and curcumin inhibited JNK activation. Notably, anisomycin, an activator of JNK, prevented curcumin-induced ubiquitination and subsequent degradation

of SIRT1. Taken together, these observations suggest that curcumin exerts anti-carcinogenic effect, at least in part, through destabilization of SIRT1 in HCT-116 cells, which is attributable to its inhibition of SIRT1 phosphorylation at the serine 27 residue.

Keywords

Colorectal cancer; Curcumin; c-Jun N-terminal kinase (JNK); Posttranslational modification; SIRT1

2. Introduction

Colorectal cancer (CRC) is one of the leading causes of cancer-related deaths worldwide, thus considered as a global public health problem (1). Accumulating evidence suggests that dietary phytochemicals found in the fruits, vegetables, herbs and spices, have the potential to prevent or delay CRC. Curcumin, a bioactive compound found in the rhizome of turmeric (*Curcuma longa* L., Zingiberaceae), has been widely investigated with regard to its chemopreventive and anticarcinogenic properties. Mechanistically, curcumin exerts anti-tumor activity through downregulation of Wnt/β-catenin in human colon cancer (SW480) cells (2). This effect was mediated through inhibition of the microRNA (miR)-130a and a subsequent increase in the mRNA/protein level of Nkd2, a negative regulator of the Wnt/β-catenin signaling pathway. It has also been reported that curcumin induces G2/M cell cycle arrest and apoptosis by suppressing Akt and promoting the cleavage of caspase-3 in cisplatin-resistant human ovarian cancer (A2780) cells (3).

SIRT1, an NAD^+-dependent class III histone deacetylase (HDAC), has been shown to delay aging and extend the life span of laboratory animals (4-6) and improve obesity and metabolic diseases (7-10). It also exerts anticarcinogenic effects in several experimental tumor models (11-13). However, recent studies have shown that SIRT1 is overexpressed in human colon cancer tissues as well as several colon cancer cell lines, suggesting that this enzyme

may function as a tumor promoter (14, 15). However, the molecular basis of pro-tumorigenic functions of SIRT1 in the progression of CRC remains largely unknown. It has been known that posttranslational modifications (PTMs) could diversify the function of proteins, and dysregulation of PTMs could contribute to acquisition of malignant characteristics of cells. Among the known PTMs of SIRT1, phosphorylation occurring at the specific amino acids has unique effects on protein stability (16-18) and catalytic activity (19-21) of this enzyme. Recently, hyperphosphorylation of SIRT1 has been observed in colorectal cancer tissues, implying the relevance of SIRT1 phosphorylation to its oncogenic functions (22).

Aberrant activation of c-Jun N-terminal kinase (JNK) has been reported to contribute to tumorigenic potential of various types of cancer cells (23-26). As one of the main components of mitogen-activated protein kinases (MAPKs), JNK responds to multiple stimuli including oxidative stress, cytokines and growth factors. Its phosphorylation-dependent activation can provoke the activation of other kinases and transcription factors involved in oncogenesis. Therefore, elaboratively controlling the JNK pathway is important to maintain the normal cellular physiology. Here, I report that curcumin destabilizes SIRT1 protein through inactivation of JNK and subsequent interruption of SIRT1 phosphorylation at the serine 27 residue in human colon cancer (HCT-116) cells, thereby inhibiting their proliferation, migration and growth.

3. Materials and methods

Reagents

Curcumin was purchased from LKT Laboratories, Inc (St. Paul, MN, USA). Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco-BRL (by Thermo Fisher Scientific Inc.; Waltham, MA, USA). MG-132 was obtained from Enzo Life Sciences (Exeter, UK). Cycloheximide (CHX) and anisomycin were supplied by Sigma-Aldrich (St. Louis, MO, USA). SP600125, U0126 and SB203580 were purchased from Tocris Bioscience (Bristol, UK).

Cell culture

HCT-116 cells were obtained from the Korean Cell Line Bank (KCLB) and maintained routinely in DMEM containing 10% FBS and a 100 ng/ml antibiotics mixture under humidified atmosphere of 5% CO_2/95% air.

Transient transfection of SIRT1-WT, SIRT1-S27A and SIRT1-S27D plasmid

Transient transfection with plasmid vectors was performed using Lipofectamine® 2000 (Invitrogen by Thermo Fisher Scientific Inc.; Waltham, MA, USA) according to the

instructions supplied by the manufacturer. After 48 h of transfection, cells were treated with curcumin alone or in combination with CHX according to the purpose of the experiment.

Western blot analysis

Lysates from cells were separated by running through 6-10% sodium dodecyl sulfate polyacrylamide gel and transferred to the polyvinylidene difluoride membranes according to the protocol described elsewhere (27). The membranes were then incubated with primary antibodies against SIRT1, phospho-p38, p38, extracellular signal regulated kinase (ERK) and actin (Santa Cruz Biotechnology, Inc.; Dallas, TX, USA), ubiquitin (Life Technolgies by Thermo Fisher Scientific Inc.; Waltham, MA, USA), phospho-SIRT1, phospho-ERK, phospho-JNK, JNK (Cell Signaling Technology; Danvers, MA, USA). Blots were washed with Tris-buffered saline with 0.1% Tween-20 and then probed with horseradish peroxidase-conjugated secondary antibodies (Pierce Biotechnology; Rockford, IL, USA). The transferred proteins were visualized by using a Western blotting detection reagent kit (AbClon; Seoul, South Korea).

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from HCT-116 cells by using the TRIzol® reagent (Invitrogen by Thermo Fisher Scientific Inc.; Waltham, MA, USA), and RT-PCR was performed according to the standard protocol. The primers used for the RT-PCR are as follows (forward and reverse, respectively): for *SIRT1*, 5′-GCAACATCTTATGATTGGCAC-3′ (position 620 to 640) and 5′-AAATACCATCCCTTGACCTGAA-3′ (position 891 to 870) with a product size of 272 bp. For *IL-1β*, 5′-GCATCCAGCTACGAATCTCC-3′ (position 229 to 248) and 5′-CCACATTCAGCACAGGACTC-3′ (position 936 to 917) with a product size of 708 bp. For *IL-6*, 5′-GTGTGAAAGCAGCAAAGAGGC-3′ (position 493 to 513) and 5′-CTGGAGGTACTCTAGGTATAC-3′ (position 652 to 632) with a product size of 160 bp. *IL-8*, 5′-ATGACTTCCAAGCTGGCCGTGGCT-3′ (position 154 to 177) and 5′-TCTCAGCCCTCTTCAAAAACTTCT-3′ (position 445 to 422) with a product size of 292 bp. For *GAPDH*, 5′-GCATGGCCTTCCGTGTCCCC-3′ (position 857 to 876) and 5′-CAATGCCAGCCCCAGCGTCA-3′ (position 1072 to 1053) with a product size of 216 bp.

Immunoprecipitation

For ubiquitination of SIRT1, cells were treated with MG-132 (20 μM) and curcumin (10 μM) or SP600125 (20 μM) for 3 h. According to purpose of the experiment, cells were exposed to MG-132 (20 μM) and curcumin (10 μM) alone or in combination with anisomycin (1 ng/ml)

for 3 h. Lysates from cells were prepared as described elsewhere (28). After resolving the samples by sodium dodecyl sulfate polyacrylamide gel electrophoresis, membranes were incubated with primary antibody to ubiquitin.

Wound healing assay

For the wound healing assay, cells were plated into culture-insert (Ibidi; Martinsried, Germany) to make a 500 μm gap. After detaching the insert from a plate, cells were grown for the indicated periods of time and then photographed under the microscope.

Clonogenic assay

For the clonogenic assay, cells were seeded in 6-well plates (200 cells/well) and grown for 14 days. Colonies were stained with 0.05% crystal violet. The number of colonies (more than 50 cells) was counted.

Statistical analysis

The statistical significance was determined using the Student's *t*-test. Differences between

groups were considered statistically significant if $p < 0.05$.

4. Results

Curcumin inhibits phosphorylation of SIRT1 at the serine 27 residue, thereby facilitating its degradation in human colon cancer (HCT-116) cells

I previously observed that curcumin, when treated to HCT-116 cells, reduced the protein level of SIRT1 but not the mRNA level, suggesting the destabilization of SIRT1 protein rather than inhibition of *de novo* synthesis of SIRT1 by curcumin (29). It has been reported that SIRT1 is stabilized by posttranslational modifications, especially phosphorylation at the serine 27 (16, 18). Based on this notion, I first determined the phosphorylation level of SIRT1 at the serine 27 following treatment of HCT-116 cells with curcumin. As shown in **Fig. 4-1A**, curcumin resulted in decreased phosphorylation of SIRT1 at the serine 27 concomitant with a decrease in the total level of SIRT1. The reduced phosphorylation might be due to phosphorylation-independent degradation of SIRT1, limiting the substrate availability for kinases. Pretreatment with a proteasome-specific inhibitor MG-132 rescued curcumin-induced loss of an unmodified form of SIRT1, but did not influence the level of phosphorylated SIRT1 **(Fig. 4-1B)**, excluding the above possibility. These results suggest that curcumin hampers the SIRT1 phosphorylation at the serine 27, thereby facilitating its degradation. To further verify the role of phosphorylation at the serine 27 residue of SIRT1 in its stabilization, serine 27 to alanine (SIRT1-S27A) or aspartic acid (SIRT1-S27D) substitution mutant were employed. SIRT1-

S27A mutant, which prevents potential phosphorylation, had shortened half-life of SIRT1 compared to that of wild-type SIRT1 (SIRT1-WT) **(Fig. 4-1C)**. Furthermore, SIRT1-S27D mutant that mimics phosphorylation status was resistant to curcumin-induced SIRT1 destabilization compared to SIRT1-WT **(Fig. 4-1D)**. These data suggest that curcumin-induced degradation of SIRT1 is associated with decreases in its phosphorylation at the serine 27 residue.

JNK appears to be a putative kinase responsible for phosphorylation of SIRT1

It has been known that various kinases are involved in the phosphorylation of SIRT1, thereby controlling the deacetylase activity or protein stability. Among them, MAPKs are associated with increased/decreased stability of SIRT1 (16, 17, 30). MAPKs consist of three main arms, JNK, ERK and p38. When HCT-116 cells were exposed to 10 μM curcumin for various time periods, curcumin reduced the phosphorylation levels of MAPKs **(Fig. 4-2A)**. To clarify which kinase(s) is/are responsible for phosphorylation of SIRT1, HCT-116 cells were treated with the SP600125 or U0126 or SB203580, which is a specific inhibitor of JNK, MEK/ERK and p38, respectively. Although each inhibitor worked well as evidenced by decreased phosphorylation of MAPKs, only SP600125 effectively diminished the levels of phospho- and total SIRT1 **(Fig. 4-2B)**. These results suggest that JNK appears to be a putative kinase

responsible for phosphorylation of SIRT1.

JNK-dependent phosphorylation of SIRT1 is responsible for its stabilization

Next, the association between JNK-dependent phosphorylation and stabilization of SIRT1 was investigated. Treatment of HCT-116 cells with SP600125 could not influence the mRNA expression of SIRT1 as assessed by RT-PCR analysis **(Fig. 4-3A)**. Given the fact that SP600125 treatment resulted in the reduction of both phospho- and total SIRT1, JNK is most likely to posttranslationally regulates SIRT1. Notably, not only curcumin but SP600125 provoked ubiquitination of SIRT1 **(Fig. 4-3B)**, thereby decreasing its half-life in the cycloheximide chase assay **(Fig. 4-3C).** These findings suggest that JNK-dependent phosphorylation of SIRT1 contributes to its stabilization.

JNK overactivation protects SIRT1 from curcumin-induced destabilization

In another experiment, HCT-116 cells were coincubated with curcumin and anisomycin, an activator of JNK. As shown in **Fig. 4-4A,** curcumin or anisomysin or combination of curcumin and anisomysin did not influence the mRNA level of SIRT1. Notably, anisomycin-induced JNK activation dampened curcumin-mediated suppression of SIRT1 as analyzed by Western

blot analysis (**Fig. 4-4B)**. This effect was achieved by attenuation of curcumin-induced ubiquitination of SIRT1 by anisomycin (**Fig. 4-4C**). In parallel with these findings, curcumin-induced inhibition of migration and growth of cells was concomitantly blunted by pharmacologic activation of JNK (**Fig. 4-4D** and **E,** respectively). These data lend further support to the notion that serine 27 phosphorylation of SIRT1 protects it from curcumin-induced destabilization.

Phosphorylation of SIRT1 enhances the oncogenicity of HCT-116 cells

I next examined the biological significance of phosphorylation of SIRT1 regarding the characteristics of cancer cells. In the wound healing assay, serine 27 to aspartic acid substitution rendered HCT-116 cells insensitive to curcumin-mediated inhibition of their migration (**Fig. 4-5A)**. In addition, colony formation of cells expressing SIRT1-S27D was affected by curcumin to a much lesser extent than cells expressing SIRT1-WT in the clonogenic assay (**Fig. 4-5B).** It has been suggested that proinflammatory cytokines produced by tumor cells could stimulate the growth and proliferation of tumor cells themselves. Furthermore, they contribute to migration and invasion of cancer cells through multiple signal transduction pathways. Among many proinflammatory cytokines, interleukin (IL)-1β, IL-6 and IL-8 have been known to promote the survival, growth and migration of colon cancer

cells (31-33). I previously checked that SIRT1 positively regulates these proinflammatory cytokines at the transcriptional level, suggesting that SIRT1 may partly contribute to the migration and growth of colon cancer cells through production of proinflammatory cytokines. This supposition is also supported by RT-PCR analysis. Again, inhibition of proinflammatory cytokine production by curcumin was significantly attenuated in cells harbouring mutant SIRT1 (SIRT1-S27D) compared with those expressing wild-type SIRT1 (SIRT1-WT) (**Fig. 4-5C)**.

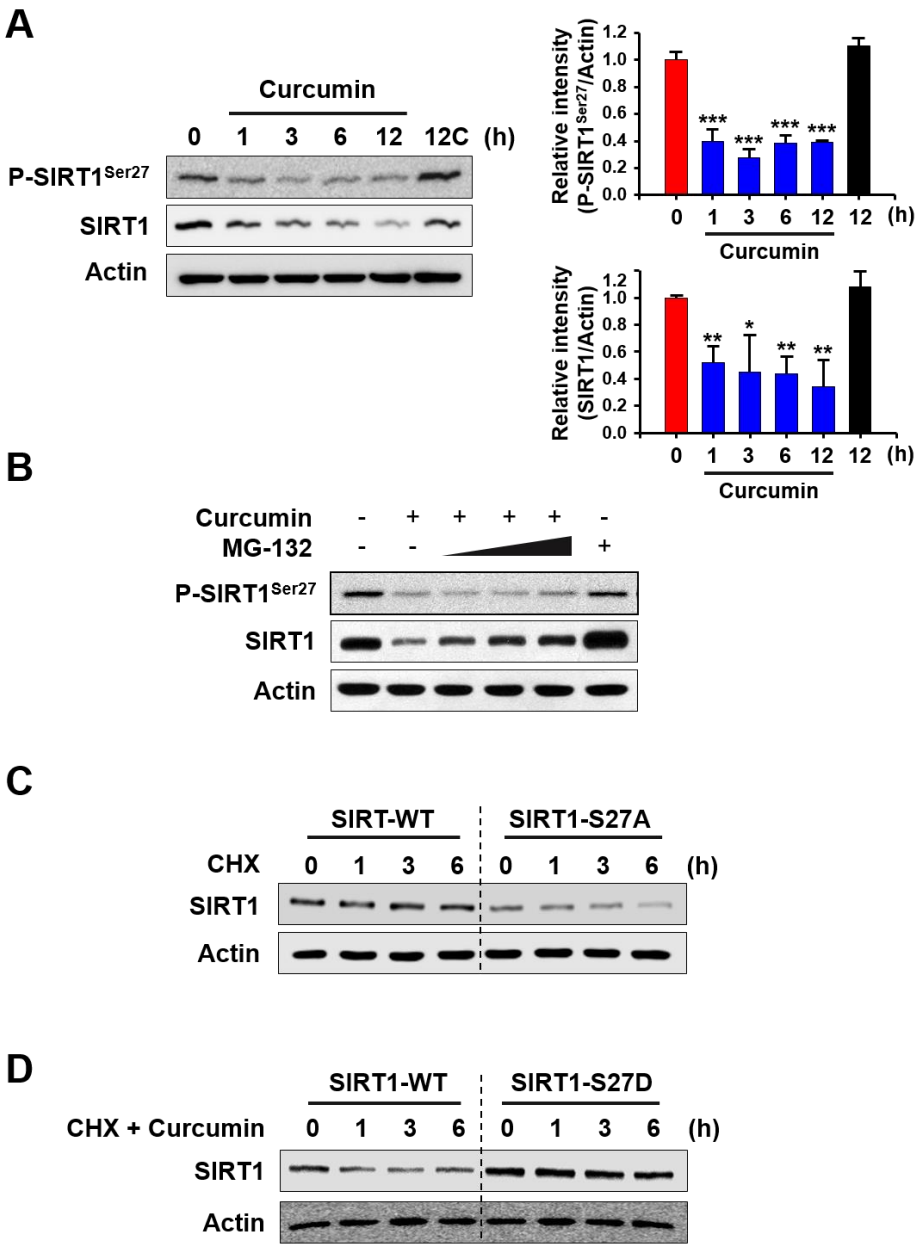


Fig. 4-1. Effects of curcumin on phosphorylation and stability of SIRT1. (A) HCT-116 cells were treated with curcumin (10 μM) for the indicated durations. The protein levels of phospho-SIRT1^{Ser27} and SIRT1 were determined by immunoblot analysis. The results are

shown as the mean ± S.D. of triplicates, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. (B) HCT-116 cells were exposed to curcumin (10 μM) alone or in combination with three different concentrations (5 or 10 or 20 μM) of the proteasome inhibitor MG-132 for 3 h, and the protein levels of phospho-SIRT1^{Ser27} and SIRT1 were examined by Western blot analysis. (C) HCT-116 cells were incubated with cycloheximide (CHX; 70 μM) for indicated time periods following transfection with wild-type (SIRT1-WT) or mutant-SIRT1 (SIRT1-S27A), and the protein level of SIRT1 remaining at each time point was determined by immunoblot analysis. (D) HCT-116 cells were treated with curcumin (10 μM) and cycloheximide (CHX; 70 μM) for indicated time periods following transfection with wild-type (SIRT1-WT) or mutant-SIRT1 (SIRT1-S27D), and the protein level of SIRT1 remaining at each time point was measured by Western blot analysis.

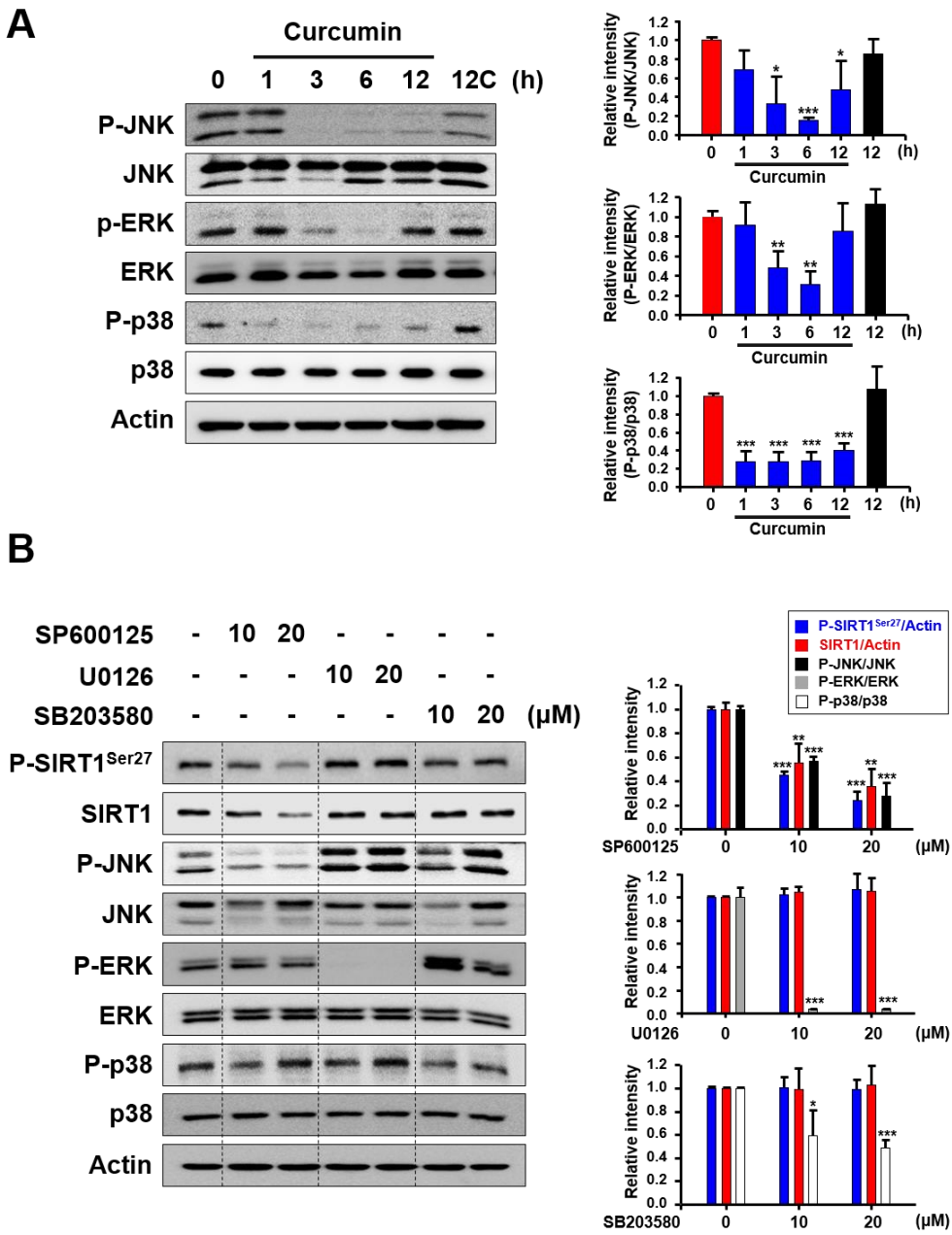


Fig. 4-2. Possible involvement of JNK in phosphorylation of SIRT1. (A) Following treatment of HCT-116 cells with curcumin (10 μM) for indicated time periods, the protein levels of phospho-JNK, JNK, phospho-ERK, ERK, phospho-p38 and p38 were examined by

immunoblot analysis. The data are presented as the mean ± S.D. of three independent experiments, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. (B) HCT-116 cells were treated with two different concentrations (10 or 20 μM) of a JNK inhibitor (SP600125), a MEK/ERK inhibitor (U0126) or a p38 inhibitor (SB203580) for 3 h. The protein levels of phospho-SIRT1^{Ser27}, SIRT1, phospho-JNK, JNK, phospho-ERK, ERK, phospho-p38 and p38 were measured by Western blot analysis. The results are shown as the mean ± S.D. of three independent experiments, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

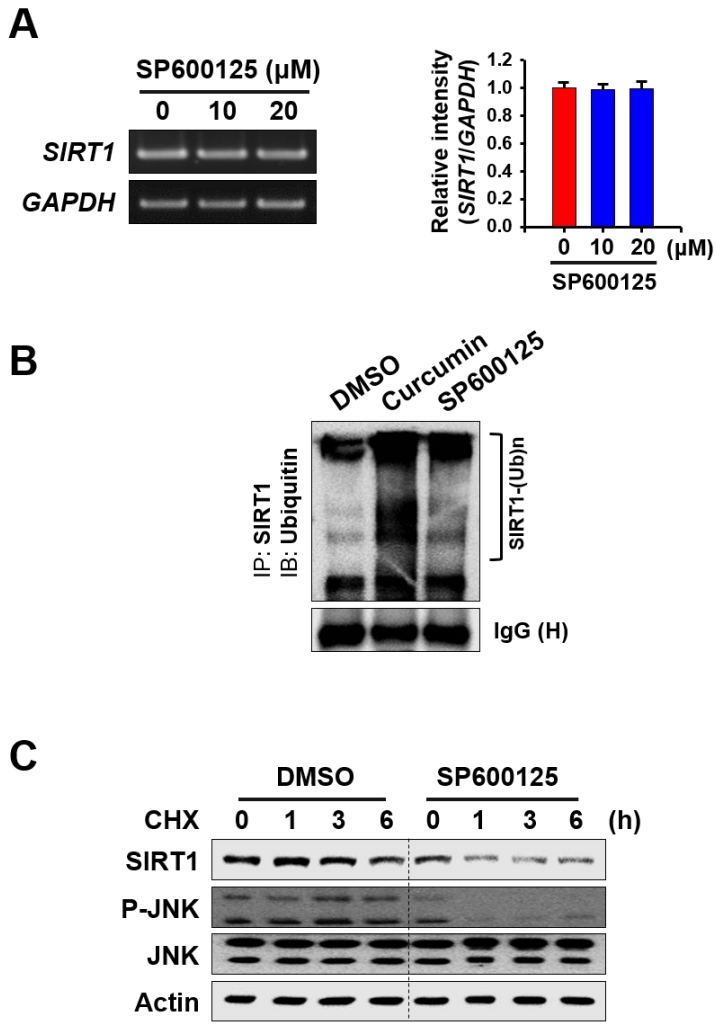
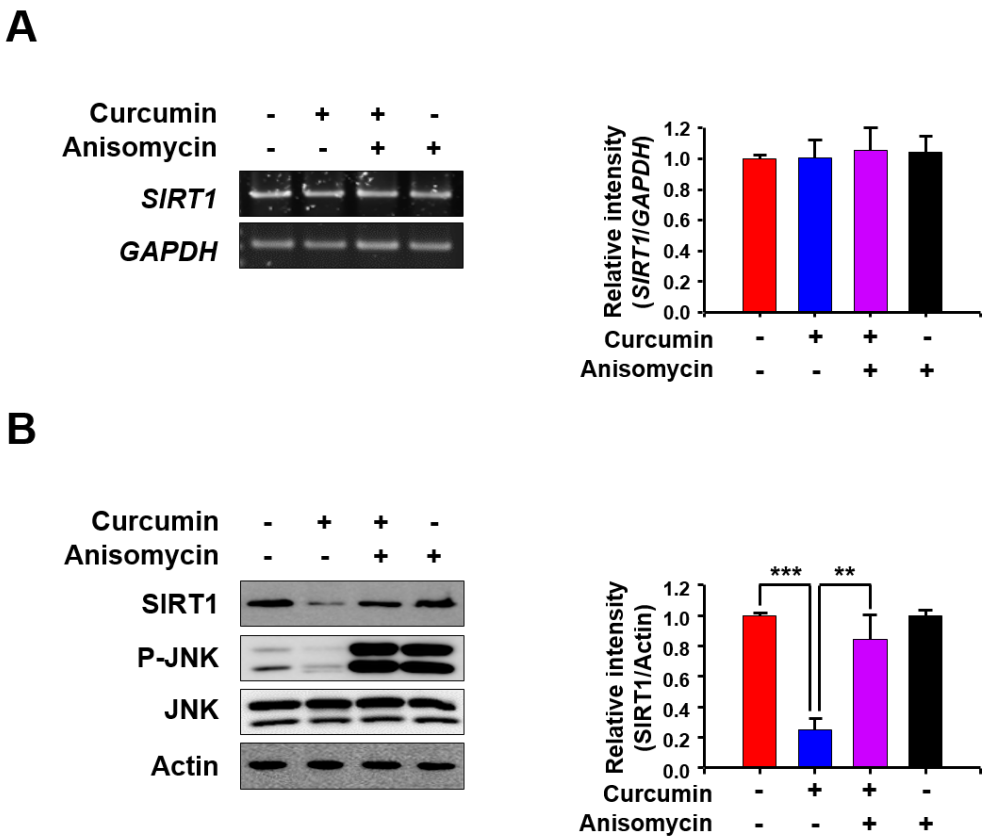


Fig. 4-3. Possible involvement of JNK in stabilization of SIRT1. (A) Following treatment of HCT-116 cells with SP600125 (10 or 20 μM) for 3 h, the mRNA level of SIRT1 was measured by RT-PCR. (B) Following treatment of HCT-116 cells with MG-132 (20 μM) and curcumin (10 μM) or SP600125 (20 μM) for 3 h, the ubiquitination of SIRT1 was measured by immunoprecipitation. (C) HCT-116 cells were treated with cycloheximide (CHX; 70 μM) for indicated periods in the absence or presence of SP600125 (20 μM).


A
Curcumin - + + -
Anisomycin - - + +
SIRT1
GAPDH
Relative intensity (SIRT1/GAPDH)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Curcumin - + + -
Anisomycin - - + +
B
Curcumin - + + -
Anisomycin - - + +
SIRT1
P-JNK
JNK
Actin
***
**
Relative intensity (SIRT1/Actin)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Curcumin - + + -
Anisomycin - - + +
C
Curcumin - + + -
Anisomycin - - + +
IP: SIRT1
IB: Ubiquitin
SIRT1-(Ub)n
IgG (H)

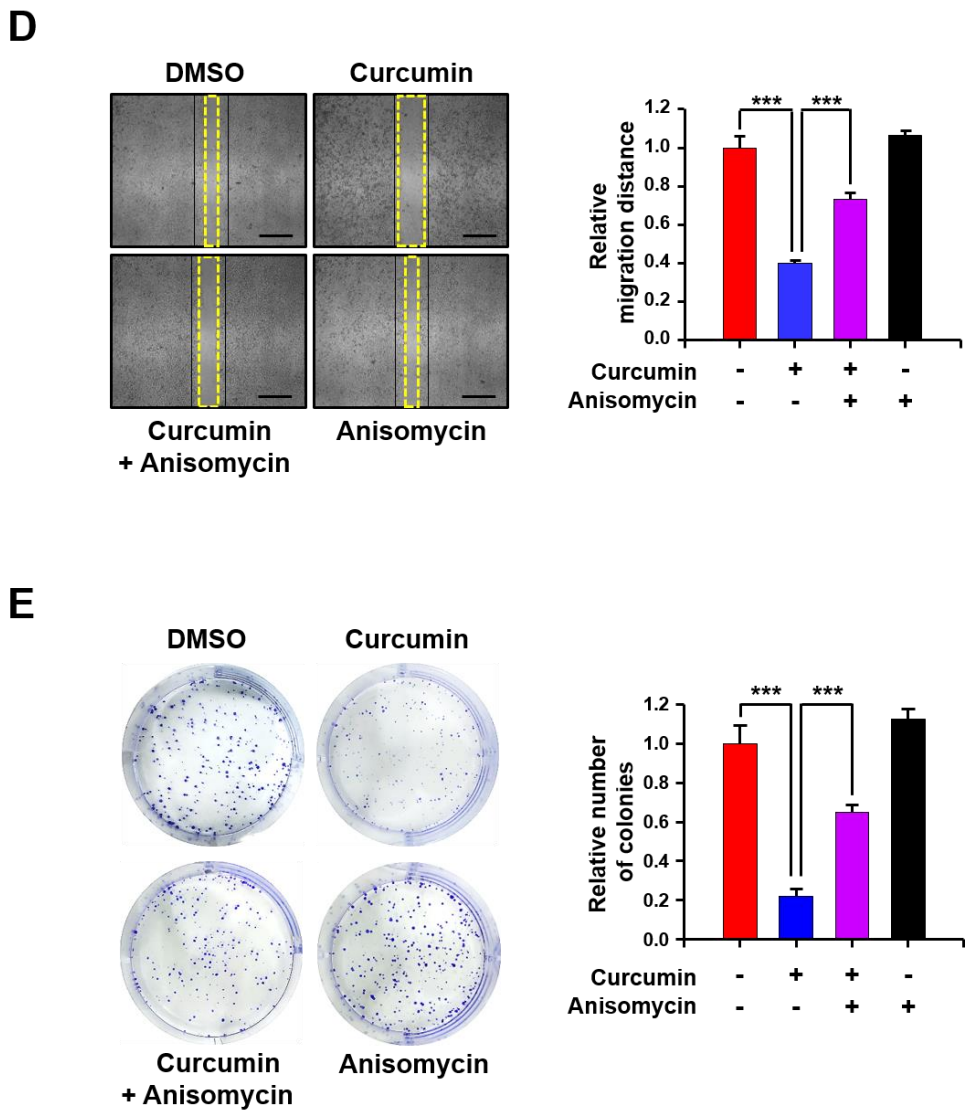
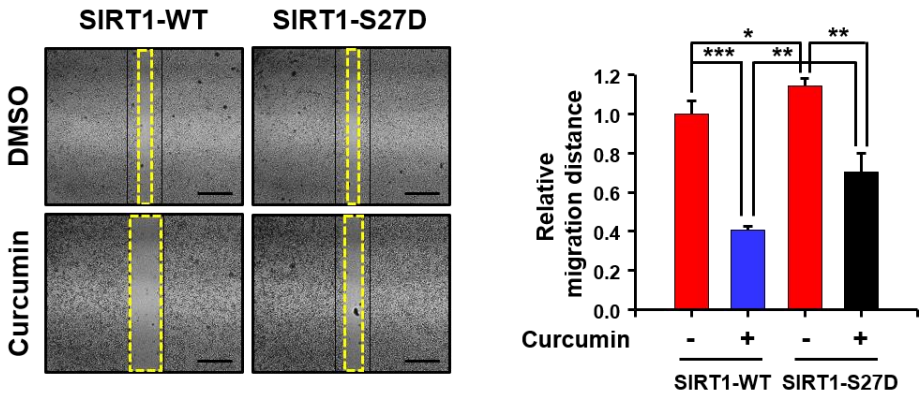


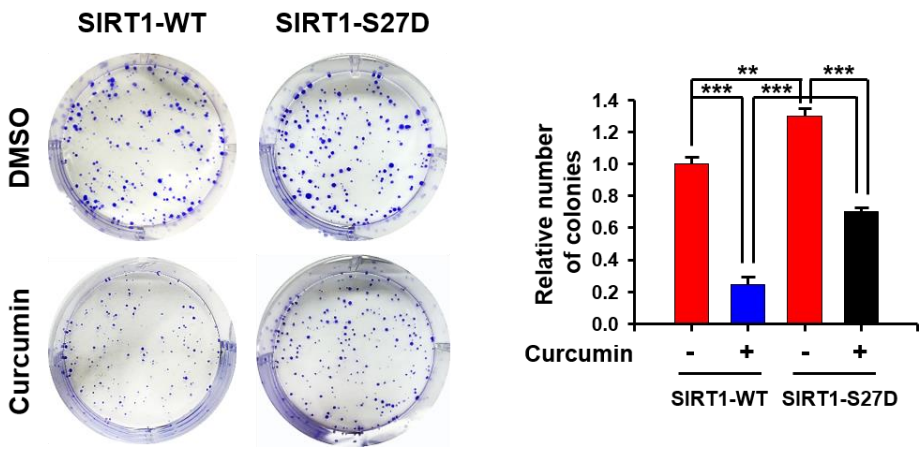
Fig. 4-4. Protection of SIRT1 against curcumin-induced destabilization by overactivation of JNK. (A, B) HCT-116 cells were exposed to curcumin (10 μM) in the presence or absence of a JNK activator anisomycin (1 ng/ml) for 3 h. The mRNA (A) and protein (B) levels of SIRT1 or JNK were measured by RT-PCR and Western blot analyses,

respectively. GAPDH and Actin were used as equal loading control for normalization. The data are presented as the mean ± S.D. of three independent experiments, $^{**}p < 0.01$, $^{***}p < 0.001$. (C) Following treatment of HCT-116 cells with MG-132 (20 μM) and curcumin (10 μM) alone or in combination with anisomycin (1 ng/ml) for 3 h, the ubiquitination of endogenous SIRT1 was assessed by immunoprecipitation. (D) HCT-116 cells were exposed to curcumin (10 μM) alone or in combination with anisomycin (1 ng/ml) for 48 h, and the cell migration was assessed. Each scale bar represents 500 μm. The values are resented as the mean ± S.D. of three independent experiments, $^{***}p < 0.001$. (E) Clonogenic efficiency of HCT-116 cells incubated with curcumin (10 μM) alone or in combination with anisomycin (1 ng/ml) was measured as described in Materials and Methods. The results are shown as the mean ± S.D. of three independent experiments, $^{***}p < 0.001$.

A


SIRT1-WT
SIRT1-S27D
DMSO
Curcumin
Relative migration distance
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
**
***
**
Curcumin
-
+
-
+
SIRT1-WT
SIRT1-S27D

B


SIRT1-WT
SIRT1-S27D
DMSO
Curcumin
Relative number of colonies
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
***
***
***
Curcumin
-
+
-
+
SIRT1-WT
SIRT1-S27D

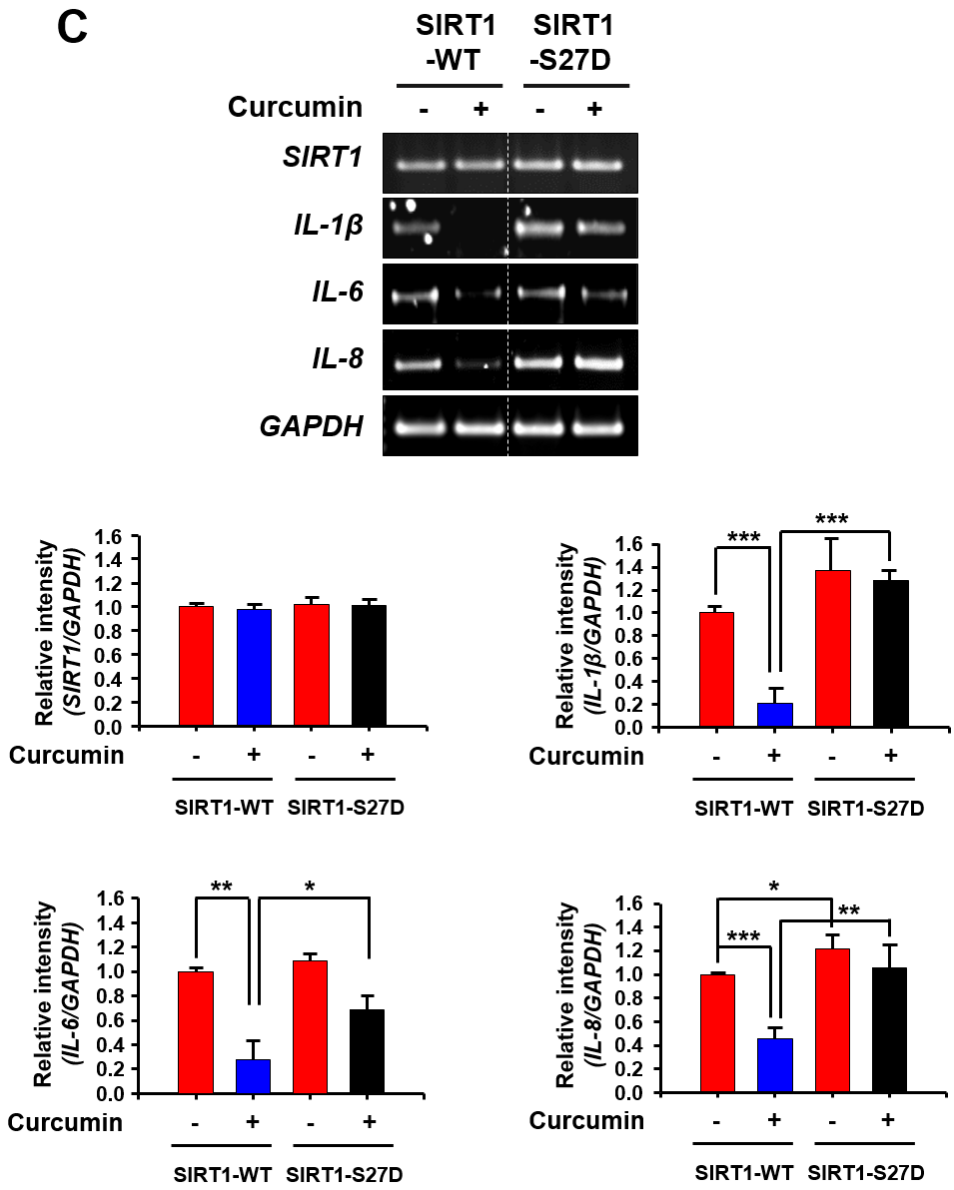


Fig. 4-5. Amelioration of the inhibitory effects of curcumin on migration and growth of HCT-116 cells harbouring mutant SIRT1 in which serine 27 is substituted by aspartic acid. (A) HCT-116 cells were transiently transfected with wild-type SIRT1 (SIRT1-WT) or serine 27 to aspartic acid mutant SIRT1 (SIRT1-S27D). Cells were then treated with curcumin

(10 μM) for the wound healing assay as described in Materials and Methods. Each scale bar represents 500 μm. The data are presented as the mean ± S.D. of three independent experiments, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. (B) HCT-116 cells were transiently transfected with SIRT1-WT or SIRT1-S27D for 48 h and were then seeded into 6-well plates, followed by curcumin (10 μM) or vehicle treatment for the clonogenic assay. The values are resented as the mean ± S.D. of three independent experiments, **$p < 0.01$, ***$p < 0.001$. (C) HCT-116 cells were transfected with SIRT1-WT or SIRT1-S27D for 48 h. Following treatment of cells with curcumin (10 μM) for 3 h, the mRNA levels of SIRT1, IL-1β, IL-6 and IL-8 were examined by RT-PCR. The results are shown as the mean ± S.D. of three independent experiments, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

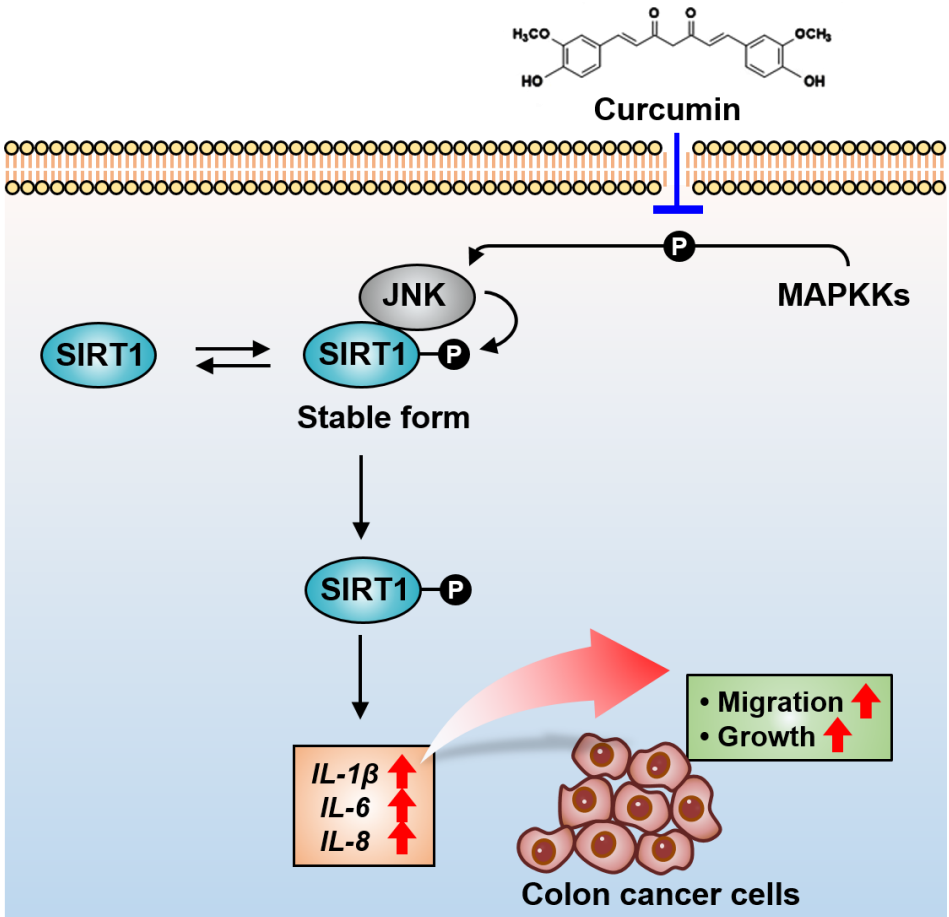


Fig. 4-6. A proposed mechanism underlying curcumin-induced destabilization of pro-tumorigenic SIRT1. Curcumin inhibits phosphorylation of serine 27 residue of SIRT1 through inactivation of JNK. The resulting unphosphorylated SIRT1 is susceptible to ubiquitin-dependent proteasomal degradation.

5. Discussion

It has been suggested that the protein levels of SIRT1 and phospho-SIRT1 are higher in colorectal cancer tissues than adjacent normal tissues (22). Abnormally overexpressed SIRT1 is associated with the advanced tumor node metastasis stage. Likewise, phospho-SIRT1 is positively correlated with a cellular proliferation marker, Ki67. Thus, phosphorylation of SIRT1 has been considered to have distinctive role in CRC.

As one of well-documented posttranslational modifications of protein, phosphorylation can alter the protein in many aspects including activity, stability and binding affinity to other proteins. To date, SIRT1 has been known to contain eighteen phosphorylation sites (19-21, 34, 35), and phosphorylation occurring at these amino acids is positively or negatively associated with protein stability and catalytic activity. Serine 27 residue of SIRT1 is phosphorylated by JNK2, resulting in the enhanced SIRT1 protein stability in human colon cancer (HCT-116) cells (36). Moreover, both serine 27 and 47 of SIRT1 can also be phosphorylated and stabilized by Ca^{2+}/calmodulin-dependent protein kinase kinase β in human umbilical vein endothelial cells (HUVECs) under pulsatile shear stress (18). In contrast, phosphorylation of mouse SIRT1 at serine 46, which corresponds to serine 47 of human SIRT1, is mediated by JNK1 and related to its ubiquitin-dependent degradation in mouse adipocytes (3T3-L1) and human embryonic kidney (HEK293) cells (37).

Meanwhile, phosphorylation occurring at other serine or threonine residues is involved in catalytic activity rather than protein stability of SIRT1. Phosphorylation of SIRT1 by AMP-activated protein kinase at threonine 344 residue led to activation of SIRT1 in human osteosarcoma (U2OS) cells (38), but the phosphorylation by this kinase at the same residue caused opposing effect in human hepatocellular carcinoma (HepG2) cells, leading to promotion of p53 acetylation as well as enhancement of apoptotic activity (39). Serine 434 of SIRT1 is susceptible to phosphorylation by protein kinase A. This modification activated SIRT1, which in turn, activated PGC-1α through deacetylation to induce the transcription of lipid metabolism genes in U2OS cells (19). Furthermore, two anti-apoptotic dual-specificity tyrosine phosphorylation-regulated kinase (DYRK) members, DYRK1A and DYRK3, activated the SIRT1 through phosphorylation at threonine 522 residue to promote the cell survival in U2OS cells (20). CK2-mediated phosphorylation of SIRT1 at serine 659 and serine 661 rendered human non-small cell lung cancer (NSCLC) (H1299) cells resistant to etoposide-induced apoptosis via deacetylation of p53, indicating the phosphorylation-dependent activation of SIRT1 (40).

Stimulation of ubiquitin-dependent degradation of proteins related to inflammation and cancer, such as cyclooxygenase-2 (COX-2) (41), ErbB2 (42) and Akt (43), has been proposed as one of the prime mechanisms underlying an anti-carcinogenic effect of curcumin. In the

current study, I demonstrated that curcumin induces degradation of SIRT1 through inhibition of JNK-dependent phosphorylation at the serine 27 residue. However, how curcumin could inhibit the activation of JNK and how unphosphorylated SIRT1 could be readily degraded remain to be elucidated.

JNK is redox-sensitive transcription factor. It has been reported that nitric oxide (NO) can suppress the activation of JNK in murine macrophage (RAW264.7) cells (44). Notably, cysteine 116 to serine substitution mutation of JNK invalidated the inhibitory effect of NO on JNK activity. In addition, JNK-IN-2, 8 and 11, the first irreversible inhibitors of JNK developed in 2012 (45), inactivate JNK via covalent modification of its conserved cysteine 116 residue (46). As curcumin has two electrophilic α,β-unsaturated carbonyl groups, it can covalently modify cysteine thiols of some proteins by Michael addition (29, 47, 48). It is hence speculated that curcumin could inactivate JNK by directly modifying the cysteine residue of JNK.

Several reports indicate that phosphorylation is a prerequisite for some proteins to interact with their interacting proteins (49, 50). In addition, it has been suggested that USP22 deubiquitinates and stabilizes SIRT1 through physical interaction with SIRT1 (51). Therefore, there might be a possibility that phosphorylation of SIRT1 enhances the interaction between SIRT1 and its endogenous stabilizer USP22 to prevent SIRT1 from ubiquitination dependent

degradation.

In conclusion, curcumin reduces protein stability of SIRT1 through suppression of JNK-dependent phosphorylation concomitant with inhibition of migration and growth of HCT-116 cells. Thus, the inhibitory effects of curcumin on progression of colon cancer are, at least partially, achieved by suppression of phosphorylation at the serine 27 residue and subsequent destabilization of oncogenic SIRT1.

6. References

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CONCLUSION

Properly controlled expression of SIRT1 is required for maintaining the human health, thus dysregulation of SIRT1 is associated with several pathophysiological conditions, such as metabolic syndrome, cardiovascular and neurodegenerative diseases. Although, there are two conflicting meta-analyses of gastrointestinal cancers regarding correlation between SIRT1 and overall survival, recent studies have indicated that SIRT1 has clinicopathological significance in CRC. Notably, the elevated levels of SIRT1 are significantly associated with tumor invasion, lymph node metastasis and higher grades of malignancy, suggesting the prognostic utility of SIRT1.

This study showed that SIRT1 is required for tumor formation *in vivo*. Silencing of SIRT1 resulted in a significant decrease in the volume and the weight of colon cancer xenograft tumors. The results from the *in vitro* assays parallel those observed in the nude mouse xenograft assay, corroborating the oncogenic function of SIRT1 in the progression of CRC. It is assumed that SIRT1 could promote the migration, invasion and growth of CRC cells by enhancing the production of proinflammatory cytokines, which is mediated through positive regulation of STAT3 protein stability by SIRT1.

The use of natural compounds derived from edible plants has been considered as an

attractive strategy to control tumor-promoting proteins. Here, curcumin, a yellow pigment present in the rhizome of turmeric (*Curcuma longa* L., Zingiberaceae), exerted the inhibitory effect on oncogenic SIRT1. Curcumin that contains the electrophilic α,β-unsaturated carbonyl moiety can directly bind to SIRT1 protein, preferentially at the cysteine 67 residue. The resulting structural modification facilitates the ubiquitin-dependent proteasomal degradation of SIRT1 overexpressed in CRC cells.

Additionally, curcumin can indirectly reduce the protein stability of SIRT1 through dephosphorylation. JNK appears to be a putative kinase responsible for phosphorylation of SIRT1, thus reduced phosphorylation of SIRT1 is mainly due to the inhibition of JNK activation by curcumin. Notably, phosphorylation of SIRT1 contributes to not only its stabilization but enhancement of the oncogenicity of HCT-116 cells.

Taken together, these direct and indirect modifications of SIRT1 by curcumin can collaboratively lead to proteasomal degradation of oncogenic SIRT1, which accounts for anti-cancer effects of curcumin in terms of suppression of cell migration and growth, and this study will provide better understanding of the potential of SIRT1 as a chemotherapeutic target for the treatment of CRC.

국 문 초 록

대장암은 전세계적으로 빈번히 발생하는 악성종양으로, 2018년에 발표된 자료에 따르면 미국의 경우 새롭게 진단된 암 발생률 뿐만 아니라 암으로 인한 사망률에 있어서도 남성과 여성 모두에서 3위를 차지하고 있다. 대장암의 위험인자로는 신체 활동의 결핍, 비만, 알코올 섭취와 함께 육류 및 포화지방이 다량 함유된 식이 등이 대두되고 있으며 이에 따라 서구식 식습관이 만연해진 한국에서도 대장암 발병률이 증가하고 있는 추세이다. 대장 점막 세포에서 발생한 유전자 돌연변이는 대장 용종을 유발하며, 그 크기가 클수록 악성 세포를 포함할 가능성이 높기 때문에 대장내시경을 통한 용종 관찰은 대장암 조기 진단에 있어서 큰 역할을 해왔다. 그러나 최근의 연구 결과들은 이러한 외과적 접근 외에도 생물학적표지인자 특히 종양특이적인 단백질을 대장암 진단 및 예후 인자로 활용하는 의료기술의 유용성에 주목하고 있다.

SIRT 단백질 계통은 7개의 이성질체로 구성되어 있으며 NAD^+ 의존적인 class Ⅲ 히스톤 탈아세틸화효소군에 속한다. 이들 이성질체는 히스톤 뿐만 아니라 다른 단백질들도 탈아세틸화 시킬 수 있으며 알려진 7개의 이성질체 가운데 SIRT1은 가장 활발히 연구 되어져 왔다. 1999년에 발표된 발아 효모의 수명 연장 효과를 첫 시작으로, 대사 조절, 분화 그리고 스트레스 반응과 같은 다

양한 현상에서의 SIRT1의 중요성이 밝혀져 왔으나, 암화과정에서의 SIRT1의 역할은 아직도 의견이 분분하다. 최근의 인체 대장암 조직 연구결과들을 살펴보면, 대장암 환자에 있어 대장암 조직 부위의 SIRT1이 인접한 정상 조직에 비해 과도하게 발현되어 있으며 이러한 과발현된 SIRT1은 종양 침윤 및 림프절 전이 뿐만 아니라, 대표적 암-관련 단백질인 *KRAS*와 *BRAF*의 돌연변이와도 상당한 관련성이 있음이 밝혀졌다.

본 연구에서도 역시 면역형광염색기법을 통해 인체 대장암 조직을 염색해보았을 때 인접 정상조직에 비해 종양 부위의 SIRT1이 과도하게 염색됨을 통해 SIRT1의 과발현을 확인하였다. 또한, 유전자 발현을 선택적으로 억제할 수 있는 small interfering RNA (siRNA) 기법을 이용해 인체 대장암 세포주(HCT-116, HCT-15, DLD-1그리고 SW480)에서 SIRT1을 선택적으로 억제하였을 때, 이들 암세포주의 특성이라 할 수 있는 활발한 세포 성장 및 이동이 유의하게 억제되었다. 뿐만 아니라, SIRT1이 선택적으로 억제된 HCT-116 세포주를 누드 마우스 피하에 이종이식 주사하였을 때 형성된 종양의 크기가 대조군 마우스에 비해 현저히 감소됨은 물론 Cyclin D1, Proliferating cell nuclear antigen (PCNA) 및 c-Myc과 같은 종양 조직 내 세포 증식인자들의 단백질 발현 역시 대조군 마우스 조직에 비해 낮아져 있음을 통해 대장암에서의 SIRT1의 역할이 종양 촉진적임을 확인할 수 있었다

이러한 암 촉진성 단백질을 제어할 수 있는 물질로서 천연 식물 유래의 화합물의 가치가 중요하게 여겨지고 있다. 흥미롭게도 2003년에 발표된 세계보건기구(World Health Organization; WHO)의 보고에 따르면 인도의 대장암 발생률이 남녀 모두에 있어서 미국에 비해 상대적으로 10배 가량 낮은 것으로 밝혀졌는데 역학 조사에 의하면 curcumin과 같은 식이성 파이토케미칼이 풍부한 인도 음식이 낮은 대장암 발병률에 기여할 것으로 여겨진다. Curcumin은 강황의 뿌리에 많이 존재하고 있는 노란색의 색소 성분으로서 화학적 암예방이나 항암효과에 관해서는 광범위하게 연구 되어져 왔으며, 세포 신호전달회로와 관련되어 있는 다양한 단백질들을 조절함으로서 뛰어난 항염증 및 항산화효과를 나타낸다. 기존의 연구들에 따르면 신경세포나 대식세포 혹은 평활근세포 등과 같은 정상세포주에서는 curcumin에 의한 SIRT1 단백질의 활성화가 세포 보호작용에 기여하는 것으로 보고되었으나 암세포주에서는 curcumin과 SIRT1의 관계가 확실히 규명된 바 없다. 따라서 본 연구는 기존의 역학조사 및 연구를 바탕으로, curcumin에 의한 SIRT1 조절 효과와 이에 의한 대장암 제어 가능성을 확인하고자 하였다. Curcumin을 인체 대장암 세포주(HCT-116, HCT-15, DLD-1 및 SW480) 에 처리하였을 시, SIRT1의 mRNA에는 영향을 미치지 못하였으나 시간 의존적으로 SIRT1의 단백질이 감소되었다. 이는 curcumin이 SIRT1의 신생 합성에 관여하는 것이 아니라 단백질 번역 후 변형(post-translational

modification)에 관여함을 암시한다. 가장 흔히 알려진 단백질 번역 후 변형 중 유비퀴틴화/탈유비퀴틴화는 단백질의 분해와 밀접한 연관이 있기 때문에 세포 내에서 특정 단백질의 수준을 일정 수준으로 유지하는 데에 큰 역할을 한다. 통상, 유비퀴틴화가 진행된 단백질의 경우 다음 단계로 단백질 분해효소 복합체인 프로테아좀(proteasome)에 의해 분해가 진행된다. 이를 토대로 curcumin이 SIRT1의 단백질 안정화를 낮추어 분해시킬 가능성에 초점을 두고 유비퀴틴화 분석을 수행한 결과, curcumin에 의해 SIRT1의 유비퀴틴화가 촉진됨을 확인할 수 있었다. Curcumin에 의해 억제된 SIRT1의 단백질 발현이 프로테아좀 억제제 처리 시 복원된 것을 통해 curcumin에 의한 SIRT1 억제는 SIRT1의 단백질 분해에 의한 것이며 이는 프로테아좀 의존적인 유비퀴틴화에 의한 것임을 알 수 있었다. 종합적으로 분석해보면 curcumin은 SIRT1을 유비퀴틴화 시킴으로서 단백질 분해효소 복합체를 통해 분해되도록 하며 결과적으로 SIRT1 단백질을 불안정화시킨다.

대장암이나 간암에서 관찰된 바에 따르면 과발현된 SIRT1은 mRNA 수준이 아니라 단백질 수준에서 비롯된 것으로서, 이는 암종에서 적절한 수준으로 조절되지 못하고 과도하게 안정화된 SIRT1이 암화 과정에 기여한다는 것을 의미한다. 그러나 그럼에도 불구하고 SIRT1의 세포내 안정화 기작에 대해서는 밝혀진 바가 많지 않다. Curcumin은 구조적 특징으로서 α,β-unsaturated

carbonyl group을 갖고 있으며 이 특징으로 인해 세포 내 단백질의 친핵성 잔기에 잘 결합할 수 있음이 보고된 바 있다. 특히, 친전자성 구조가 결핍되어 있는 curcumin 유도체인 tetrahydrocurcumin의 경우 위의 언급된 SIRT1에 대한 curcumin의 효과와 동일한 효과를 내지 못한 점을 비추어 볼 때, SIRT1 단백질 불안정화 및 분해를 유도함에 있어서 curcumin의 구조적 특징인 $\alpha,\beta-$unsaturated carbonyl group이 중요하며, 이 부위를 통해 SIRT1 단백질 내 특정 친핵성 잔기에 직접 결합할 수 있는 가능성이 있음이 제시된다. 따라서 이를 근거로 질량분석법(mass spectrometry analysis)을 실시한 결과, SIRT1의 친핵성 잔기인 cysteine 67번에 curcumin이 직접 결합할 가능성이 높음을 확인하였다. 특정 부위 돌연변이(site-directed mutagenesis)를 통해 cysteine 67번을 alanine으로 치환했을 경우, curcumin과 SIRT1간의 결합이 저해됨과 더불어 SIRT1의 분해 역시 일어나지 않은 현상은 curcumin이 SIRT1의 cysteine 67번 잔기에 결합하여 분해를 유도함을 반증한다. 뿐만 아니라 cysteine 67번이 alanine으로 치환되었을 시, 대장암 세포 이동 및 성장이 curcumin 처리에도 불구하고 크게 영향을 받지 않은 현상을 통해 curcumin은 직접 결합에 의해 SIRT1을 분해함으로써 암세포 이동 및 성장 억제와 같은 항암효과를 나타낸다는 것을 알 수 있었다.

2015년에 발표된 임상 조직 결과는 대장암 환자 조직에서 SIRT1 뿐만 아

니라 인산화된 형태의 SIRT1 역시 과도하게 발현되어 있으며 단백질의 번역 후 변형 형태 중 하나인 인산화에 의해 SIRT1 단백질의 안정화가 높게 유지될 가능성을 제시하였다. 이는 암화 과정에 관여하는 단백질 인산화효소인 JNK에 의해 SIRT1의 인산화 및 단백질 안정화가 촉진된다는 연구결과에 의해서도 뒷받침된다. 이러한 문헌 정보를 바탕으로 curcumin에 의한 JNK 활성 억제가 SIRT1의 탈인산화 및 단백질 분해를 유도할 가능성을 도출하였고, 사전적으로 curcumin에 의해서 JNK 활성화 및 SIRT1 인산화가 시간의존적으로 저해됨을 확인하였다.

JNK에 의한 SIRT1 인산화가 실제로 SIRT1의 단백질 안정화에 영향을 미치는지 확인하기위해 HCT-116 세포주에 JNK 억제제를 처리하였을 때, SIRT1 mRNA 수준에는 큰 변화가 없었지만 단백질 수준은 떨어진 것을 통해 JNK가 단백질 번역 후 변형 단계를 통해 SIRT1에 영향을 미친다는 것을 알 수 있었다. 단백질 신생 합성 억제제 처리를 통해 새롭게 합성되는 SIRT1 단백질을 저해한 조건에서, 기존에 존재한 SIRT1 단백질의 분해가 JNK 억제제 처리에 의해 대조군에 비해 더 촉진되는 것을 확인할 수 있었고, 이는 JNK가 SIRT1 단백질의 안정화에 기여한다는 것을 의미한다. 이와 유사하게 JNK 활성제 처리 시 curcumin에 의해 억제되었던 SIRT1 발현이 회복됨과 동시에 SIRT1의 유비퀴틴화는 억제된 것은 curcumin에 의한 SIRT1 유비퀴틴화 및

단백질 분해는 JNK 억제에 의해 매개된 것임을 의미한다.

실험에 사용된 대장암 세포주에서의 SIRT1 발현은 해당 세포의 이동성이나 성장에 있어서 맥락을 같이 하는 것으로 보여진다. 세포 이동을 확인할 수 있는 wound healing assay를 시행하였을 때, curcumin에 의해 억제되었던 세포 이동이 JNK 활성제 처리 시 상쇄됨을 알 수 있었고 이러한 현상은 세포 성장을 확인할 수 있는 clonogenic assay에서도 동일하게 관찰되었다. 이는 대장 암세포 내에서의 curcumin 작용이 JNK에 의해서 상쇄된다는 것을 의미하는데 한 가지 주목할 만한 점은 JNK 활성제 단독처리 시 대조군과 비교했을 때 암세포가 가지는 특성이 특별히 더 강화되지는 않았다는 점이다. 이는 아마도 해당 암세포 내에 이미 JNK가 활성화되어 있어 추가적인 상승효과를 내지는 못하였으나 curcumin 처리와 같은 자극적인 상황에서는 활성제에 의한 JNK 활성화가 암세포에 대한 보호 작용을 나타낼 수 있으며 분자적으로 이는 JNK 활성화에 의해 SIRT1 단백질의 분해가 방해되는 것과 관련이 있을 것으로 사료된다.

JNK가 단백질 인산화효소라는 점을 고려하면 SIRT1 단백질 안정화는 SIRT1 인산화와 관련이 있을 것으로 사료되는데, 이는 JNK에 의해서 인산화되는 잔기라 여겨지는 SIRT1의 serine 27 잔기를 인산화 모방성을 띄는 aspartic acid로 치환하였을 때 대조군에 비해 SIRT1 단백질 수준이 더 높게 유지된 점과, curcumin 처리시에도 덜 예민하게 반응한 결과를 통해서도 유추할 수 있다.

Serine 27번 잔기를 인산화가 불가능한 alanine으로 치환한 경우, SIRT1의 반감기가 대조군에 비해서 더 급격히 떨어진 현상 또한 이를 뒷받침한다. 앞서 SIRT1 발현이 해당 암세포의 이동성 및 성장에 유의한 영향을 미친 것을 참고로, SIRT1의 serine 27번 잔기가 aspartic acid로 치환된 세포의 이동성 및 성장 능력을 측정한 결과, 대조군에 비해 세포 이동성이나 성장 능력에 있어서 우월성을 갖고 있음을 wound healing assay 및 clonogenic assay를 통해 확인하였다.

이상의 결과들을 종합하면 curcumin은 SIRT1 잔기 결합을 통한 직접적 제어를 통해서도 혹은 SIRT1의 상위 조절인자 억제를 통한 간접적 제어를 통해서도 SIRT1을 억제하는 효과를 나타낼 수 있음을 알 수 있다. 이러한 직 · 간접적인 SIRT1 억제는 대장암 세포주의 이동 및 성장을 저해했을 뿐만 아니라, 누드 마우스 이종이식 모델에서의 종양 성장 역시 저해하였다. 본 연구를 통해 제시된 SIRT1의 암 촉진적 기능에 주목해볼 때 향후 대장암 진단 및 치료를 위한 새로운 표적으로서 SIRT1의 가능성이 제시되며, 항암화학요법의 효과를 높이기 위한 보조제로 curcumin과 같은 천연물질을 사용하고자 하는 시도들의 귀추가 주목된다.

Key words

Colorectal cancer; SIRT1; Curcumin; Thiol modification; c-Jun N-terminal kinase; Phosphorylation

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