이학박사 학위논문

Regulation of sirtuin 6 protein expression by cyclic AMP signaling system in non-small cell lung cancer cells

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Regulation of sirtuin 6 protein expression by cyclic AMP signaling system in non-small cell lung cancer cells

A thesis submitted by

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Abstract

Cyclic AMP (cAMP) signaling system regulates a variety of cellular functions including metabolism, gene expression, cell proliferation, and death. However, the role of the cAMP signaling to regulate epigenetic change, especially including histone modifying proteins, is poorly studied.

SIRT6 is one of sirtuin families and has NAD^+ dependent histone deacetylase (HDAC) activity. SIRT6 removes acetyl group at lysine 9 and 56 from histone H3, and regulates glucose homeostasis, genomic stability and cell viability, but the regulation of SIRT6 activity is not clearly understood. Thus, in order to clarify the mechanism by which the cAMP signaling controls histone modifying proteins in non-small cell lung cancer, we made a hypothesis that cAMP can modulate the SIRT6 activity to regulate radiation-induced apoptosis of lung cancer cells. We investigated the effect of the cAMP signaling on the expression of SIRT6 protein and γ-radiation-induced apoptosis of lung cancer cells. Activation of cAMP signaling by expression of constitutively active Gαs (GαsQL),

treatment with Gαs-coupled receptor agonists such as prostaglandin E2 (PGE2) and isoproterenol, or treatment with forskolin reduced SIRT6 protein expression without change in its mRNA level in H1299 and A549 human non-small cell lung cancer lines. cAMP signaling stimulated degradation of SIRT6 protein, inhibition of proteasome with MG132 abolished the effect of cAMP signaling, and cAMP signaling increased ubiquitylation of SIRT6. Inhibition of PKA by treatment with H89 or expression of dominant negative PKA abolished the SIRT6 reduction by cAMP signaling. Treatment with PGE2 inhibited activation c-Raf by increasing inhibitory phosphorylation at serine 259 and by decreasing activating phosphorylation at serine 388 in PKA-dependent manner, which resulted in inhibition of downstream MEK-ERK signaling. Inhibition of Erk by treatment with chemical inhibitors or expression of dominant negative Erks reduced SIRT6 expression, and activation of Erk by expressing constitutively active Erk abolished SIRT6 reducing effect of PGE2. cAMP signaling augmented radiation-induced apoptosis of lung cancer cells, and exogenous expression of SIRT6 abolished apoptosis-augmenting effect of cAMP signaling. We conclude that cAMP signaling systems reduces SIRT6 expression by promoting its ubiquitin-proteasome

dependent degradation, that the degradation is mediated by PKA-dependent inhibition of ERK pathway, and that the reduced SIRT6 expression contributes to the augmented radiation-induced apoptosis by cAMP signaling in lung cancer cells.

Key Words : SIRT6, epigenetics, cAMP signaling, histone deacetylase (HDAC), PKA, c-Raf/Mek/Erk signaling, apoptosis

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Introduction

1. Guanine nucleotide-binding proteins (G protein)

G proteins are all of proteins which have enzymatic function to bind guanine nucleotides GDP and GTP. There are constituted two classes of G protein, such as monomeric small GTPases, and heterotrimeric G protein. Small G proteins are sometimes called Ras superfamily, and there are known about a hundred proteins. The Ras superfamily has nine main familes, which is involved in Arf, Rab, Rad, Ran, Rap, Ras, Rheb, Rho, and Rit (arrange alphabetically). In general, the Ras family controls cell proliferation, Rho regulates cell morphology, Ran is mediated by nuclear transport, and Rab and Arf act to control vesicle transport[1].

However, commonly G protein means the membrane-associated heterotrimeric G protein, sometimes is called 'large' G protein. These proteins are made up of three subunits, alpha(α), beta(β), and gamma(γ), and activated by G-protein-coupled receptors(GPCR) by external ligands. In human, about 1,000 GPCRs are contained in genome wide, but each protein's function is not

cleared. 21 Gα subunits, main activator in heterotrimeric G protein, are divided into four main classes, such as Gαs, Gαi, Gαq, and Gα12, and 5 β subunits and 12 γ subunits stable bind each other, so there are generally called βγ complex[1].

All of G proteins have GTPase activities, which are activated to replace from GDP to GTP by guanine nucleotide exchange factors (GEFs), or inactivated to release phospho-group of GTP by GTPase-activating protein (GAPs). When they bind GTP, signals turn on, and when they bind GDP, signals turn off. The GDP-GTP cycle is regulated by GEFs and GAPs through binding of GTP or GDP(Figure 1)[2].

1.1 Heterotrimeric G protein

When a ligand is recognized on the surface of the cell, ligand induces, and activates heterotrimeric G proteins through GPCR. In inactive state, α, β, γ subunits are bound at tri-complex state, and GDP is attached to the active site of Gα-subunit. When a ligand is bound, GDP is released, Gβγ complex is dissociated from Gα-subunit and GTP binds to GDP removing place in Gα-subunit. Both

Gα-subunit and Gβγ complex activate downstream molecules in the cells. Gαs accumulates concentration of cyclic AMP (cAMP) through adenylyl cyclase activation, whereas Gαi decreases cAMP levels by inhibition of adenylyl cyclase, and Gαq activates phospholipase C (PLC), which divides phosphatidylinositol bisphosphate (PIP_2) into diacylglycerol and inositol triphosphate(IP_3). Gα12 regulates cell mobility through activation of Rho pathways. Gβγ complex activates many signaling molecules, such as, phospholipases, lipid kinases, and ion channels (Figure 2) [3].

1.2 Cyclic adenosine 3',5'-monophosphate (cAMP)

cAMP is a first founded second messenger, acts crucial rule in the cellular organism involved in differentiation, secretion, migration, cell proliferation, and apoptosis. cAMP is created from ATP by adenylyl cyclase located on cell inner surface membrane which is activated by stimulatory GTP-binding proteins by various hormones and nuerotransmitters. Removing of cAMP to AMP is catalyzed by phosphodiesterases (PDEs). cAMP controls cAMP-dependent protein kinase A (PKA), guanine-nucleotide exchange

proteins activated by cAMP (EPAC), and cyclic-nucleotide-gated ion channels (CNG).

1.3 Cyclic AMP-dependent protein kinase A (PKA)

PKA is 170 kDa holoenzyme, and activated by cellular levels of cAMP. PKA is heterotetramer constituted by two catalytic (C) subunits, and regulatory (R) subunit dimer, and R subunits dimerize by N-terminal coiled-coil motif. The substrate recognizing site of the C subunit is bound pseudosubstrate motif in the R subunit and blocked its activity. Each regulatory subunit has two binding sites of cAMP. When increasing levels of cAMP, 4 cAMP molecules bind with two sites each 2 regulatory subunits, lead conformational change of regulatory subunit, and 2 catalytic subunits dissociate from regulatory subunits, and play active state to downstream molecules. Phosphorylation of Thr197 is required by maintain of PKA activation state. The C subunits recognize serine/threonine residues, Arg-Arg-Ser/Thr, Arg-Lys-Ser/Thr, Lys-Arg-X-Ser/Thr, or Lys-Lys-X-Ser/Thr, and attaches phosphate group to target residues (Figure 3) [4].

1.4 Cyclic AMP response element-binding protein (CREB)

CREB is well known transcription factor in nucleus and it was first reported in 1987 as a cAMP-responsive transcription factor. It is activated by PKA, and bound phosphorylation at Ser133. Activated CREB binds to cAMP response elements (CRE), and then regulates transcription level of downstream molecules. Activated CREB mostly recognizes palindromic(TGACGTCA) or half site(TGACG or CGTCA) sequences, although variable functional sites have been described. About 5,000 genes, sometimes expected one-quarter of the mammalian genome, mediated CRE element, which is regulated by CREB protein (Figure 4)[5].

2. Epigenetic change

The epigenetics means heritable changes in gene expression without changing of DNA sequence. Unlike simple genetics depend on information of the DNA sequence, the changes in phenotype or transcription/translation of epigenetics are mediated including age, the environment, and disease state. In cellular level, epigenetics is found, and researched by DNA methylation, or non-coding

RNA(ncRNA)-associated gene silencing, and histone modification field. DNA methylation means that methyl-group is attached to the cytosine or adenine residue of DNA sequence. When attached methyl-group, transcription is suppressed through block of transcription factor binding, in the nucleus. Histones are packaging protein of DNA in the cell, and there regulate gene transcription with DNA binding affinity.

2.1 Chromatin modification

Chromatin is complex form in the cell nucleus, consisting of DNA, protein (specially histone), and RNA. The nucleosome is composed of 4 histone proteins (H2A, H2B, H3, and H4), which is located in nucleo core, and each histone proteins complex is wrapped 147 base pairs of DNA sequence. Octamer complex shape of histone is globular protein except to N-terminal tail part. A specific character of histone is involved in the ability of the large number of modified residues. Recently, there are eight type of modifications found on histone, such as acetylation, methylation of Lyr, methylation of Arg, ubiquitylation, sumoylation, phosphorylation, ADP-ribosylation, Pro isomerization, and deamination. Each modification of histone tail

contributes to regulate gene expression except to changing of DNA sequence. For example, when increasing of global acetylation of histone tail, transcription level of target protein is increased in the cellular level. However, there are not mediated histone tail to modify, and each modification mechanisms of histone protein are still veiled (Figure 5) [6].

2.2 Histone acetyltransferase (HAT), Histone deacetylase (HDAC)

HAT acts to attach acetyl-group to lysine residues of each histone tails by transferring from acetyl CoA to ε-N-acetyllysine. Acetylation of histone tails increases activation of global transcription level in variable cells. HAT is divided into three families, MYST, GNAT, and CBP/p300. In general, HAT proteins have ability to modify more than one lysine, but some enzymes are shown one site specific activity. Recently, Rtt109 HAT protein acetylates a lysine-residue within the histone core part at H3(Lyr56), no tail of histone in yeast.

HDAC has reversal activity of acetylation that is related on transcriptional suppression. HDAC is divided into three families: the

HDAC class I and HDAC class II and class III NAD^+-dependent Sir family. HDAC proteins also have to remove acetyl group except to specificity, however some protein can dissociate acetyl group from lysine residue at specific site, such as SIRT6 or SIRT7. SIRT6 removes acetyl-group from histone H3 Lyr56, and SIRT7 is mediated H3 Lyr18 deacetylation [6].

2.3 Histone deacetylase inhibitors (HDACi)

HATs, or HDACs have been mediated to regulate several signaling pathways, and linked pathogenesis of cancer or other diseases, so HDACi was expected surprising tools to treat variable diseases including cancer. If someone is found that investigated-HDACi activates many apoptotic genes in specific cancer cells, HDACi is more useful to treat variable cancers. After finding of sodium butyrate, has deactylation activity, many drugs are investigated, trichostatin A (TSA), SAHA(vorinostat), tubacin, and LBH589 (panobinostat) [7].

2.4 Sirtuin (HDAC class III)

The protein, sirtuin or Sir2, is first studied in 1991 by Leonard Guarente in MIT. Sir2 is found at yeast (*Saccharomyces cerevisiae*), and formal name is 'silent mating-type information regulation 2.' Sirtuin proteins exist 2 types of Sir2 in bacteria, or archaea, and 7 types of sirtuin in eukaryote, and sirtuins are very conserved protein from an evolutionary perspective. Sirtuin proteins are made up of SIRT1 - 7: SIRT1, 6, 7 are localized in nucleus, SIRT7 is localized in cytosol, and SIRT3, 4, 5 are localized in mitochondria.

In general, sirtuin proteins are HDAC class III, and they have NAD^+- dependent histone deacetylase activities at lysine residue of histone tails. Mechanisms of deacetylation by sirtuin are showing that sirtuin proteins use one molecule of NAD^+ to deacetylate of acetyl-lysine, and final products are nicotinamide and O-acetyl-ADP-ribose at lysine reside of protein. SIRT4, 6 have different activity which is ADP-ribosyl-transferase. (SIRT4 has only ADP-ribosyl-transferase activity, but SIRT6 have both of them)

2.5 Silent mating type information regulation 2 homolog 6 (SIRT6)

SIRT6 protein belongs to HDAC class III, and specially SIRT

class IVa. For years ago, SIRT6 was expected to have only ADP-ribosyl-transferase activity, because of low activity of deacetylase. (its ability is about 1,000 times lower than SIRT1) However, SIRT6 has site specific deacetylase at histone H3 Lyr56 residue, whereas other HDAC proteins. Activation of SIRT6 has biological function in genomic stability/DNA repair, inflammation, and glucose/lipid metabolism, and variable diseases: heart hypertrophy, diabetes, obesity, cancer and aging. Recently report shows that SIRT6 contributes to regulate DNA double strand break (DSB) through C-terminal binding protein (CBP) interacting protein (CtIP), and to accelerate DNA repair by deacetylation of PARP1 or regulation of DNA-PKcs and Ku70/80. SIRT6 also blocks transcription factors involved in NF-κB, and HIF-1α. Some factors regulate expression of SIRT6 protein, although it is a little known. Similar to SIRT1, SIRT6 is also regulated by calorie restriction [8].

3. Mitogen-activated protein kinases (MAPKs) signaling pathway

MAPKs belong to serine/threonine protein kinases which is highly

conserved signaling molecules in eukaryotes. MAPKs are crucial to maintain a biological phenomenon involved in stress signal, immune system, proliferation, and apoptosis. MAPKs have variable function in cellular level, such as learning, memory, differentiation, development, and secretion of signaling molecules. In mammalian cells, MAPKs are divided three main pathways: the extracellular-signal-regulated kinase (ERK) pathway, and the p38 (synonymous name is MAPK14) pathway, and the JUN N-terminal kinase (JNK; synonymous name is MAPK8). MAPK signaling is started from variable membrane receptors at cell surface. MAPK signaling cascades are progressed by sequential phosphorylation events, MAPK kinase kinases (MAPKKKs) initiate signal transduction to attach phospho-group at two serine residues of MAPK kinases (MAPKKs). Activated MAPKKs also transfer phospho-group at the Thr/Tyr residues of MAPKs protein, active sites are constituted in Thr-X-Tyr motif (X amino acids are different between MAPKs. ERK has Glu, JNK has Pro, or p38 has Gly). Activated MAPKs regulate to transfer phosphor-group many related-downstream molecules, involved in protein kinases, and MAPK responsible transcription factors. MAPKs also control gene expression to maintain translation, mRNA stability, and transport in the cells.

These signaling cascades are mediated by occurring of many diseases, including cancer, atherosclerosis, diabetes, arthritis, and septic shock [9].

3.1 Extracellular-signal-related kinases (ERKs)

ERKs are widely expressed and well known protein kinase intracellular signaling molecules. ERKs act several biological function including meiosis, mitosis, and differentiation in the cells. ERK/MAPK cascade is mostly initiated by ligand binding to receptor tyrosine kinases (RTKs) which activates small GTPase RAS. This step is mediated by recruitment of a protein complex including the RAS exchange factor son-of sevenless(SOS) and the growth-factor-receptor bound protein-2 (GRB2) at Tyr phosphate docking site on the RTKs. Activated RAS phosphorylates the MAPKKK RAF, and activated RAF then phosphorylates the MAPKK MEK. Finally, activated MEK phosphorylates at the two site of targeted residues of the MAPK ERK. Activated ERK enters the nucleus, and controls gene expression through activation of transcription factors, and phosphorylation (Figure 6) [10].

4. Apoptosis

Apoptosis is a highly complicated, and conserved mechanism to preserve life of organ and position of each cells in multicellular organisms involved in cell compartment. Apoptosis is sometimes called 'programmed cell death' which was first referred by Currie in 1972. In all living thing, apoptosis is commonly found one of the cell death involved in morphological characteristics, and it phenomenon is observed in various cells, and tissues. In multicellular organisms, apoptosis is an essential element to perform formation of organs, and maintaining of tissues. During development, unneeded cells experience programmed cell death which important to be formed organs and tissues. If mechanisms of apoptosis fail in the cells, any organs accept to progress pathological condition, such as developmental failures, neurodegeneration, autoimmune diseases, or cancer.

The process of progress of the apoptotic signaling pathways are divided in 4 steps, including 'initiation, mediation, execution, and regulation' of apoptosis. Studying of apoptosis is started from researches in the nematode (scientific name is *Caenorhabditis*

elegans), because of most simple organ in the multicellular organisms. 131 cells are eliminated by apoptosis during development in 1,090 somatic cells. Seven genes have been identified to mediate apoptosis in nematodes. When CED-3 (caspase homologue) binds CED-4 (Apaf-1 homologue), CED-3 is activated. In normal condition, CED-4 is inactivated by binding with CED-9 (anti-apoptosis Bcl-2 homologue). EGL-1 (BH3 only member homologue) is activator to progress cell death. EGL-1 blocks CED-9 binding with CED-4, and supports to bind between CED-3, and CED-4. Components about apoptotic mechanisms are similar to all of organs, although processes are more complicated in higher organs. In mammals, activation of apoptosis are divided 2 major pathways: the intrinsic pathway (the mitochondrial pathway), or the extrinsic pathway (death receptor pathway) [11].

4.1 Intrinsic pathway

The intrinsic pathway is mediated by chemotherapeutic agents, irradiation exposures, and these process is mediated to activate BCL-2 homology 3 (BH3)-only B-cell lymphoma 2 (BCL-2)

family proteins (BCL-2 antagonist of cell death (BAD), BH3-interacting domain death agonist (BID), BCL-2-interacting mediator of cell death(BIM), p53 upregulated modulator of apoptosis (PUMA), or NOXA), and to neutralize anti-apoptotic BCL-2 proteins (myeloid leukemia cell differentiation 1 (MCL1), BCL-2, and BCL-extra large (BCL-XL)). Mitochondrial membrane is destructed by BCL families, and it acts to release cytochrome *c*, second mitochondrial activator of caspases (SMAC), and apoptosome-dependent activation of caspase-9 into the cytosol. These molecules control to activate caspase-3, and 7, and finally, they proceed to apoptosis.

4.2 Extrinsic pathway

Extrinsic pathway is death receptor dependent pathway in the cells. Death ligand, such as CD95 ligand (CD95L; also known as FASL), APO2 ligand (APO2L; also known as TRAIL), or tumor necrosis factor-α (TNF-α) bind their paired receptors to cause the formation of death-inducing signaling complex (DISC), and initiation of the extrinsic pathway. DISC recruitment activates caspase-8 and also increase activation of caspase-3, and 7.

Several ubiquitin ligases are mediated in the inhibition of cell death, including inhibitor of apoptosis proteins (IAP), whereas other E3 ligases, such as FBW7 (F-box- and WD repeat-containing 7), and MULE (MCL1 ubiquitin ligase E3), promote apoptosis (Figure 7) [12].

4.3 Ionizing radiation (IR) and apoptosis

To treat cancer patients, γ-irradiation therapy is widespread method, especially, difficult to remove tumor by surgery. Radiation therapy uses in this point that ionizing radiation breaks normal DNA strand, ultimately, inhibits growth rate of tumor cells. Radiation is special amongst DNA-damaging agents. IR generates reactive oxygen species (ROS) at localized regions in the chromosome. IR contributes base modification about 80%, and damage to the sugar phosphate backbone. One Gy of IR changes about 2,000 base modification, the formation of 1,000 DNA single-strand breaks (SSBs), and 35 double-strand breaks (DSBs) per cell. Today, it is accepted that DSBs are main cytotoxic parts in IR exposure. DSBs are repaired by non-homologous end joining (NHEJ), or homologous recombination (HR), however too highly DSBs promote

IR-induced apoptosis. IR are expected to contribute active apoptosis through DSBs, specially 'clean' DSBs act effectively to trigger apoptosis, but to find mechanism between DSBs and apoptosis is many difficult because of the broad spectrum of damages by IR exposure [13].

4.4 Treatment of lung cancer

To promote radiosensitivity, management of lung cancer is combined-modality treatment with surgery, radiotherapy, and chemotherapy by regulation of signal transduction. Tyrosine kinase inhibitors (EGFR-TKis) was developed, such as Gefitinib (Iressa), and erlotinib (Tarceva), because of EGFR mutations are well founded about 62% of NSCLC cases [14], however the EGFR gene in tumor samples showed mutation by tolerance [15]. Lately, epigenetic changed-method, which regulates broad regulation of gene expression, and variable signaling pathways by modifying histone proteins, is highly favored instead of targeted therapy. Histone deacetylase inhibitors (HDACi), one of the epigenetic changing drugs, are using anti-cancer drug by control of transcription level of tumor suppressor genes, or oncogenes [16].

SAHA (vorinostat), LBH589 (panobinostat), and valproic acid are developed to treat anti-cancer drug by inhibition of histone deacetylation.

4.5 cAMP, HDAC, and apoptosis

cAMP signaling system acts double-faced effect in variable cancers. For example, elevation of cAMP increases cell proliferation in kidney cells, hepatocytes, thyroid cell, and pc12 cells[17-19]. However, cAMP proceeds apoptosis as tumor suppressor in many various tissues, such as ovarian cancer, and lymphoma cells[20, 21]. In our experiment, we found that activation of cAMP also increases radiation-induced apoptosis in non-small cell lung cancer cells[22]. Meanwhile, acetylation of histone tail increases global transcription involved in pro-apoptotic bcl families, and proceeds apoptosis, so many researcher are developing HDAC inhibitor to respond cancer cells[23]. Although some HDAC inhibitors are developed in the recent years, inhibitors of NAD^+ dependent HDAC are less developed among HDACi, and developed drugs normally have SIRT1, or SIRT2 selective activities[24]. In the investigation for non-small cell lung carcinoma in nonsmoking women, SIRT6 is

highly expressed in lung cancer cells compare with normal tissue[25]. Thus, we focused about regulation of sirtuin by cAMP signaling system in lung cancer cells.

5. General introduction

Lung cancer is a malignant tumor arising from the respiratory epithelium, and a leading cause of cancer death in both males and females in the world. The incidence of lung cancer is second highest and still arising in woman and developing countries. Lung cancer is divided into two morphologic groups: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), and NSCLC occupies about 85% of all lung cancer [26]. Lung cancer is treated by surgery, irradiation, and chemotherapy, but the prognosis for patients with lung cancer remains dismal, with a five-year survival rate for NSCLC patients remains low about 15% [27]. Thus, intensive investigation has been made to improve the efficiency of the treatment modalities including chemotherapy and radiotherapy on the base of new understanding on the molecular mechanism of cell proliferation and death, which is anticipated to bring substantial medical benefits such as longer survival or amelioration of

symptoms [28].

3',5'-Cyclic adenosine monophosphate (cAMP) is a second messenger that activates signaling pathways regulating a variety of cellular functions including metabolism, gene transcription, cell proliferation, migration, and death. cAMP is synthesized from ATP by adenylate cyclases (ACs) which is activated by stimulatory GTP-binding proteins following stimulation of cells by numerous hormones and neurotransmitters, and cAMP is degraded into 5'-AMP by cyclic nucleotide phosphodiesterases (PDEs). cAMP can bind and activate cAMP-dependent protein kinase (PKA), guanine-nucleotide exchange proteins activated by cAMP (EPAC), and cyclic-nucleotide-gated ion channels (CNG)[29]. Activated PKA phosphorylates enzymes to regulate metabolisms and transcription factors such as cyclic AMP response element binding protein (CREB) to regulate the expression genes [30]. cAMP signaling has also been found to modulate cancer cell death including lung cancer cells induced by anticancer drugs and γ-ray [22, 31]

Histone acetylation is one of the major mechanisms for epigenetic regulation of gene expression, and results in loosening of the tightly packed heterochromatin to more relaxed euchromatin

state. The acetylation status is determined by the balance between acetylation by histone acetyltransferases (HATs) and deacetylation by histone deacetylases (HDACs) [32]. Sirtuin family is one of the four histone deacetylase (HDAC) families, and uses nicotinamide adenine dinucleotide (NAD^+) as a co-substrate. Mammals have seven sirtuin isoforms, which have different substrate specificities and subcellular localizations. The sirtuins are involved in a variety of cellular functions including genomic stability, life span, aging, energy metabolism, and tumorigenesis [33]. Among them, sirtuin 6 (SIRT6) removes acetyl group from histone H3 lysine 9, and 56 residues and regulates broad biological function including glucose homeostasis, genomic stability and apoptosis by controlling gene expression involving transcription factors such as NF-κB, and HIF-1α [34] [35] [36]. Recently, increased SIRT6 expression has been reported in various cancers including non-small cell lung cancer [25], and thus SIRT6 is implicated in carcinogenesis and tumor progression.

Both the cAMP signaling and SIRT6 have been reported to be involved in the regulation of cancer cell death evoked by anticancer drugs and ionizing radiation [37], and cAMP signaling was found to

decrease the expression of SIRT6 in our preliminary experiments. Thus, we made a hypothesis that cAMP can modulate the SIRT6 activity to regulate radiation-evoked death of lung cancer cells, and investigated the effect and the underlying mechanism of the cAMP signaling on the expression of SIRT6 protein and γ-radiation induced apoptosis of lung cancer cells. In this paper, we report that cAMP signaling reduced SIRT6 expression by stimulating its ubiquitin dependent degradation, which is mediated by PKA-Raf-/MEK-ERK pathways, and that cAMP signaling increases radiation-induced apoptosis of lung cancer cells partly by down-regulation of SIRT6 protein.

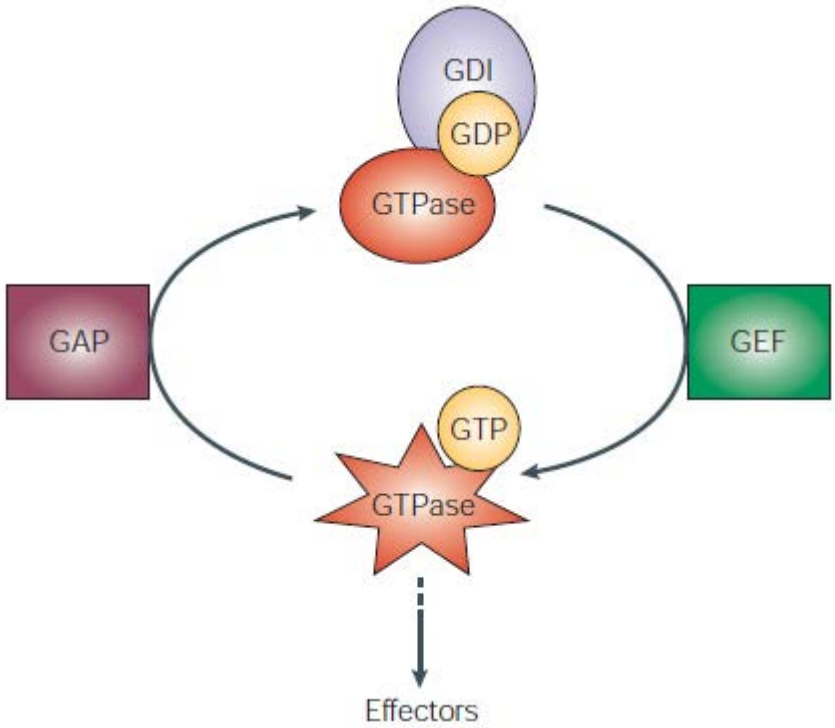

Figure 1. The GTPase cycle[38]

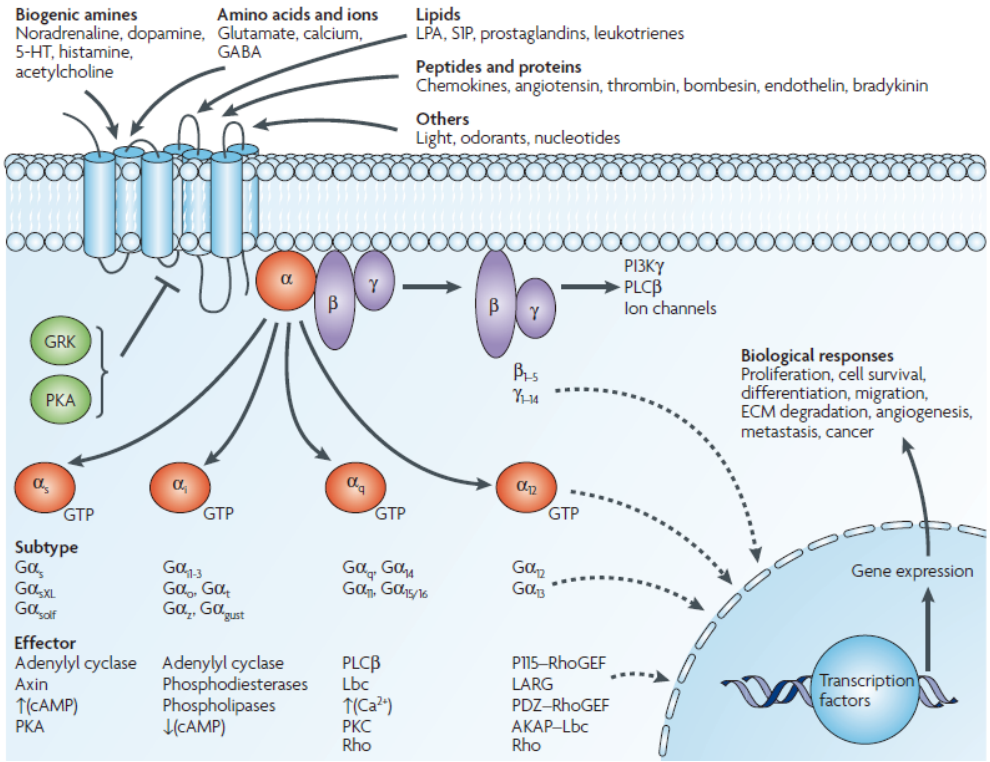

Figure 2. Diversity of G-protein-coupled receptor signaling[3]

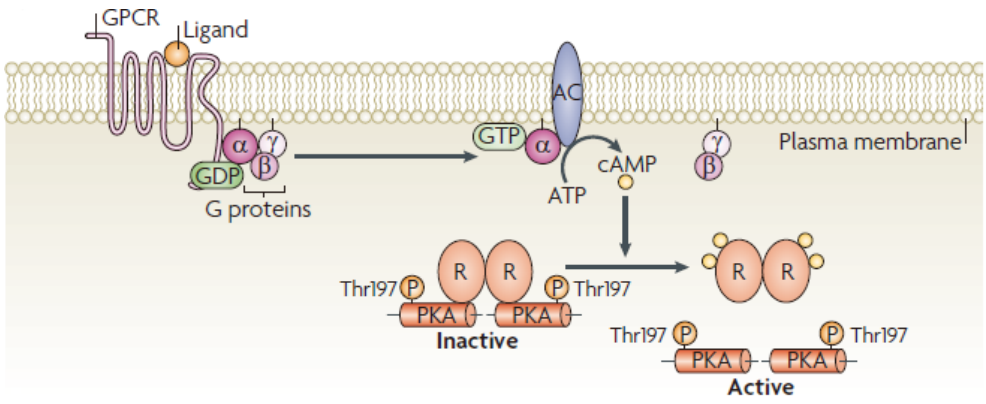

Figure 3. Mechanisms of activation of PKA [4]

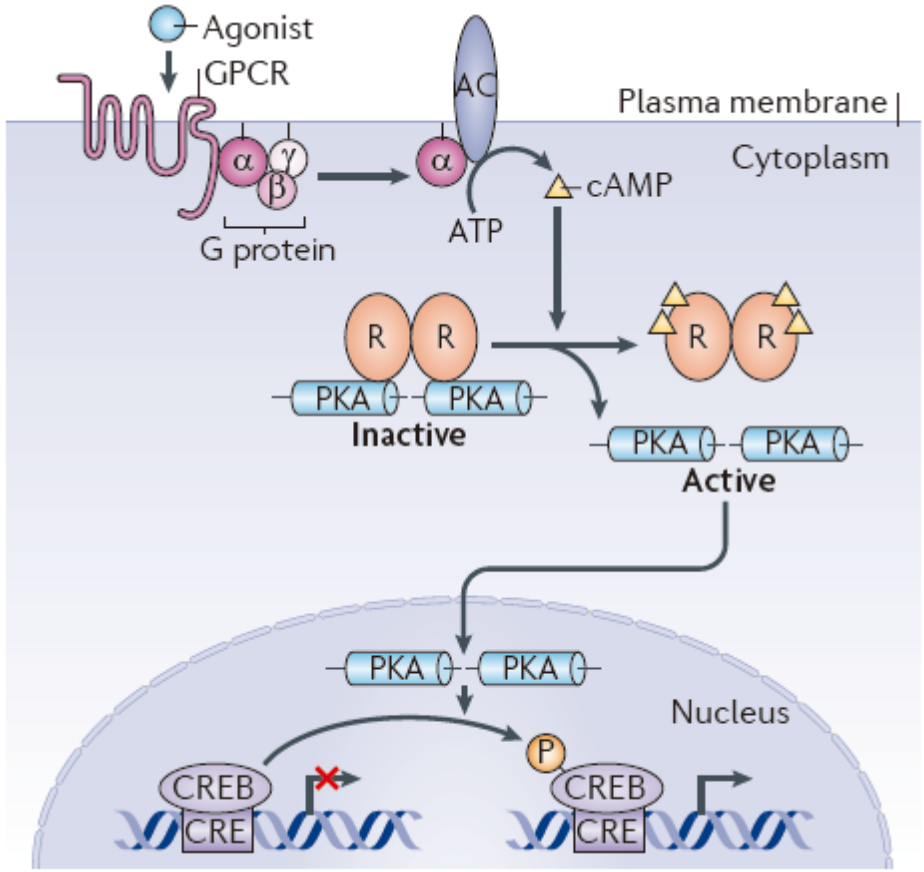

Figure 4. Mechanisms of Gαs/cAMP signaling pathway [5]

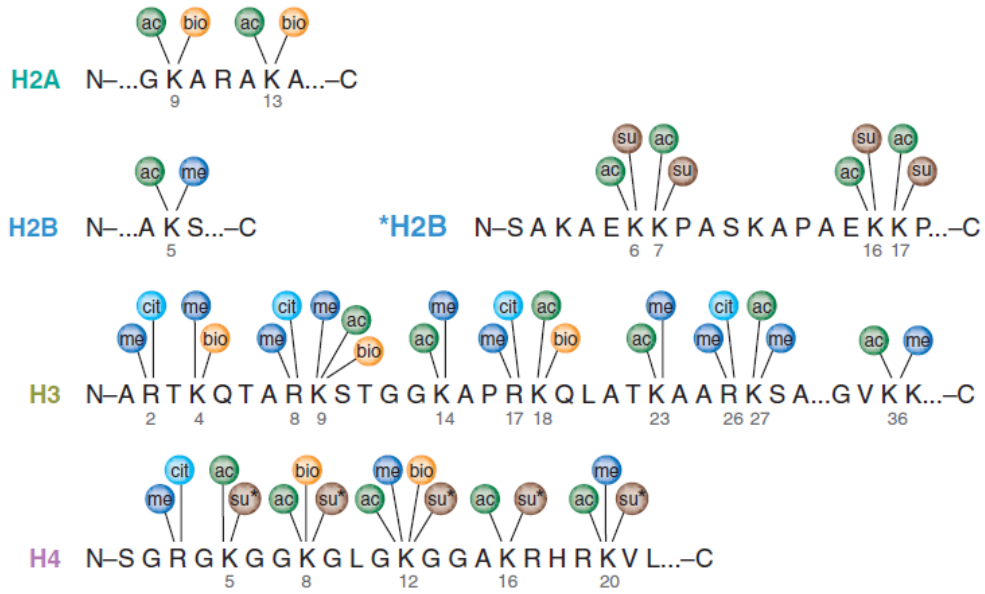

Figure 5. Variable modification in histone residues [39]

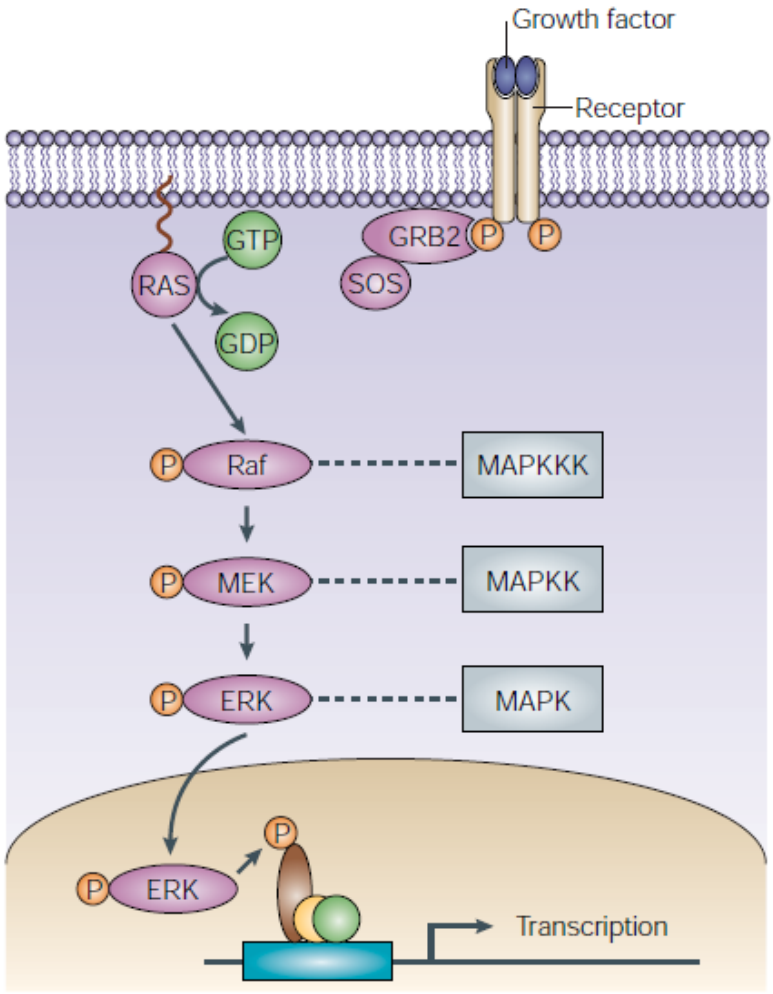

Figure 6. The ERK/MAPK cascade [10]

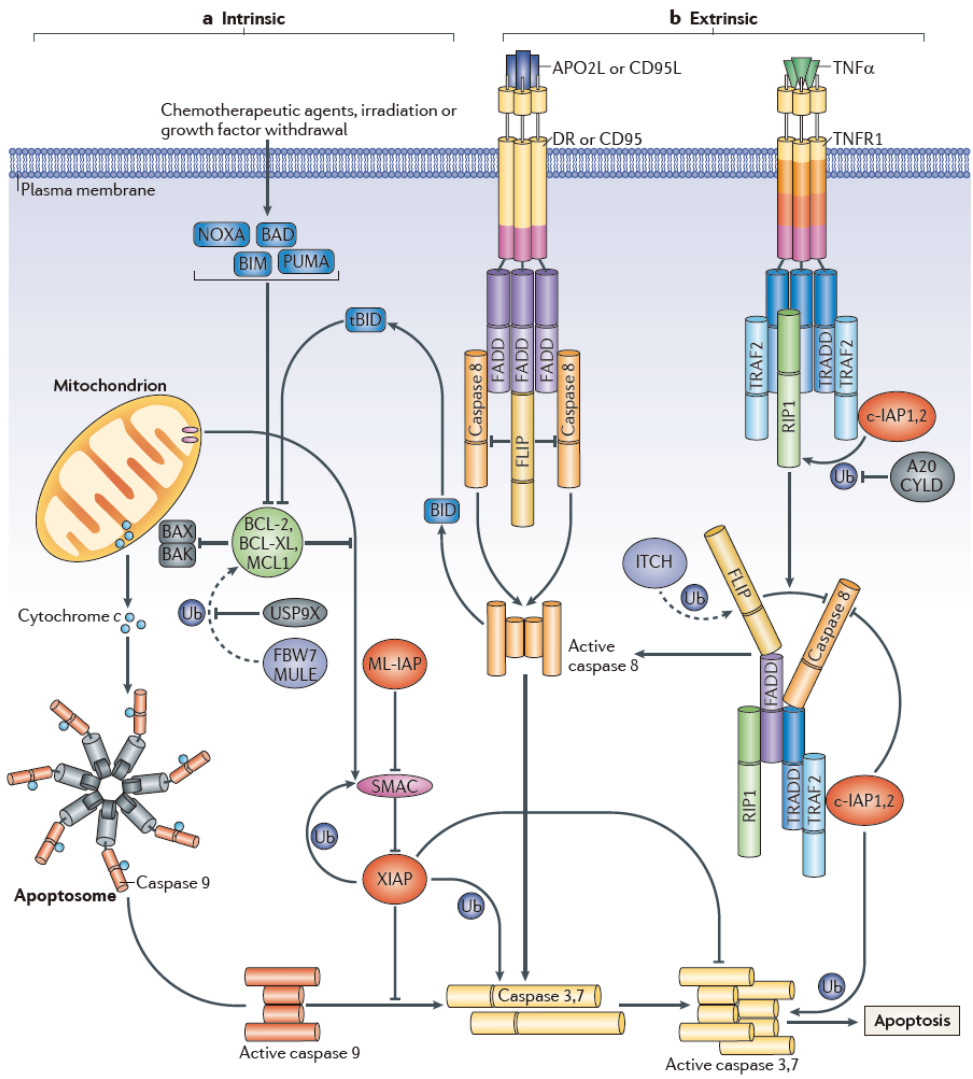

Figure 7. The intrinsic and extrinsic apoptotic pathways [12]

Purpose

Activation of cAMP increases radiation-induced apoptosis in non-small cell lung cancer cells[22]. So, it is important to find potential usage of cAMP signaling to enhance the therapeutic efficiency of radiation therapy in lung cancer cells. Understanding of the mechanisms how cAMP increases radiation-induced apoptosis in non-small cell lung cancer cells might contribute to development of targeting cAMP signaling in lung cancer treatment.

Regulation of acetylation at histone tail also occurs apoptotic changes in variable cancer cells[23]. However, regulation of acetylation at histone by cAMP signaling is poorly understood. Thus, we investigated regulation of sirtuins by cAMP signaling system in lung cancer cells.

The specific aim of this study was:

1. To investigate the effect of cAMP signaling system on the expression of SIRT6 and its underlying mechanisms in non-small cell lung cancer cells.

2. To examine the effect of SIRT6 expression on γ-ray-induced apoptosis in non-small cell lung cancer cells.

Materials and Methods

Cell culture and reagents

Human non-small cell lung cancer cell lines: H1299, and A549 (Korea Cell line Bank, Seoul, Korea) were cultured in Dulbecco's modified Eagle's medium (DMEM), RPMI-1640 containing 10% fetal bovine serum (JBI, Korea), and 100 units/ml penicillin/streptomycin. The cells were incubated in 5% CO2 incubator at 37℃. Isoproterenol, SP600125, PD0325901, 6-benzoyl-cAMP (6-Bnz-cAMP), and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Prostaglandin E2 (PGE2), forskolin, MG132, PD98059, and SB600125 were purchased from Cayman Chemical (Ann Arbor, MI, USA). The FITC Annexin V apoptosis detection kit was purchased from BD Biosciences (San Diego, CA, USA). Phenylmethanesulfonyl floride (PMSF), a protease inhibitor, sodium orthovanadate, and sodium fluoride were purchased from Roche Molecular Biochemicals (Indianapolis, IN, USA). Pyrrolidine dithiocarbamate (PDTC), IKK inhibitor VII, and BAY 11-7082 were purchased from Calbiochem (La Jolla, CA, USA), and Ku-55933 was purchased from Selleck Chemicals (Houston, TX, USA).

Transient expression of genes by transfection

H1299 and A549 cells were transfected with a constitutively active mutant of long form Gαs containing Glu-Glu-tag (GαsQ227L-EE, GαsQL-EE) in a pcDNA3.1 vector (Missouri S&T cDNA Resource Center, MO, USA) using the calcium phosphate transfection method. This mutant contains a leucine residue instead of glutamine that is essential for the intrinsic GTPase activity [40]. A dominant negative mutant of PKA (dnPKA) was a gift from Dr. G. Stanley McKnight (University of Washington, WA, USA) [41]. Wild type and dominant negative CREBs (S133A, R287L) were gift from Dr. Sahng-June Kwak (Dankook University, Cungchunganm-do, Korea). Constitutively active MEK1 was gift from Dr. Pann-Ghill Suh (Ulsan National Institute of Science and Technology, Ulsan, Korea) [42]. Wild type and dominant negative ERK1, and ERK2 (ERK1, ERK1 K71R, ERK2, ERK2 K52R) were gift from Dr. Melanie H. Cobb (University of Texas) [43]. Wild type and dominant negative SIRT6 were gift from Dr. Kartin F. Chua (Stanford University) [44]. The plasmids containing short hairpin RNA sequences targeting Gαs, CHIP (carboxyl terminus of Hsp70-interacting protein), ITCH, MDM2, Skp2, and scrambled shRNA as a negative control were purchased from Sigma-Aldrich. shRNA iduna was gift from Dr. Valina L. Dawson, and Dr. Ted M. Dawson (Johns Hopkins University) [45]. Decoy oligonucleotides for the cAMP

response element (CRE decoy) were prepared as described previously [46].

Western blot analysis

Western blotting was performed as previously described [46]. Antibodies against SIRT1, 2, 7, Skp2, Ubiquitin, and p-CREB were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and antibodies against SIRT6, CHIP, Mdm2, p-c-Raf, c-Raf, p-MEK, MEK, p-ERK, ERK, CREB, p-p38, p38, p-Jnk, Jnk, PARP, Caspase-3, and flag were purchased from Cell Signaling Technology (Beverly, MA, USA). An antibody against Glu-Glu-tag was purchased from Covance (Princeton, NJ, USA), and antibody against β-actin was purchased from Sigma-Aldrich. The proteins were visualized using the Enhanced Chemiluminescence (ECL) reagent (Advansta, Menlo Park, CA, USA) and blot images were detected by LAS-3000 luminescent image analyzer (Fuji, Tokyo, Japan). Protein densities were quantified using the Multi Gauge v.2.3 software from Fuji and calculated the ratio of control densities.

Quantitative reverse transcription polymerase chain reaction (qPCR)

qPCR was performed using a C1000 thermal cycler (BioRad, Hercules, CA, USA) as previously described [47]. The sequences

of primers used for qPCR were as follows. SIRT6, 5'-CCAAGTTCGACACCACCTTT-3', and 5'-CGGACGTACTGCGTCTTACA-3'; CHIP, 5'AGGCCAAGCACGACAAGTACAT-3', and 5'-CTGATCTTGCCACACAGGTAGT-3'; Iduna, 5'-CTACAAACAGGAAAGCGAAC-3', and 5'-CATGAAGCTCCTTTTACACA-3'; ITCH, 5'-CCTTACGTAGAGGTCACAGTAG-3', and 5'-CTCCAAGCTGCAAAGTCAC-3'; MDM2, 5'-AATCATCGGACTCAGGTACA-3', and 5'-GTCAGCTAAGGAAATTTCAGG-3'; Skp2, 5'-CTGTCTCAGTGTTCCAAGTTGCA-3', and 5'-CAGAACACCCAGAAAGGTTAAGT-3'; GAPDH, 5'-ACCACAGTCCATGCCATCAC-3', and 5'-TCCACCACCCTGTTGCTGTA-3'. After 40 cycles of PCR, the average threshold cycle (Ct) values obtained from triplicate qPCR reactions were normalized against Ct values for GAPDH.

Immunoprecipitation

H1299 cells transfected with GαsQL or vector were lysed in a lysis buffer containing 0.5% NP-40, 20 mM Tris-Cl pH7.5, 150 mM NaCl, 2 mM EDTA, 2mM EGTA, 1 mM Na_3VO_4, 1 mM NaF, 1

mM phenylmethylsulfonyl floride, and a protease inhibitor mixture. The cell lysate (800 μg of protein) was precleared by protein G agarose (Santa Cruz) for 1 h, followed by centrifugation. The supernatant were incubated with 1 μg of antibodies at 4oC overnight on a rotator, and then 40 μl of protein G agarose for 2 h before precipitate the proteins by centrifugation. The precipitate were washed 3 times before western blot analysis using ubiquitin antibody.

Flow cytometry

Lung cancer cells were exposed to γ-ray (10 Gy) and incubated for 24 h. Then, the cells were washed 2 times with phosphate-buffered saline, harvested by trypsinization, and centrifuged at 3,500 g for 5 min at 4℃. The cells were incubated in Annexin V buffer containing Annexin V antibody and PI for 15 min. The florescence of the incubated cells was detected by Facscalibur flow cytometer (BD biosciences, Franklin, NJ, USA) using 10,000 cells per sample

Data analysis

All experiments were performed at least 3 times independently,

and the data were represented as the means ± standard error (SE). The data were analyzed by non-parametric Mann-whitney U test methods, and a p value of less than 0.05 was considered statistically significant.

Results

1. cAMP signaling reduced SIRT6 expression in lung cancer cells.

To examine the effect of cAMP signaling on the expression of sirtuins, a constitutively active GαsQL was expressed to activate cAMP signaling in H1299 non-small cell lung cancer cells by transient transfection, and expression of sirtuin isoforms was analyzed by western blot analysis. The expression of GαsQL reduced the SIRT6 protein level, but increased SIRT7 protein level (Figure 8). Knock down of Gαs with shRNA increased SIRT6 expression in H1299 cells (Figure 9). Activation of cAMP signaling by treatment with Gαs-coupled receptor agonists: PGE2 and isoproterenol, or adenylyl cyclase activator forskolin also reduced SIRT6 expression in H1299 cells (Figure 10). Expression of GαsQL also reduced SIRT6 protein in A549 lung cancer cells (Figure 11). Although the number of cell-line sample was too small, SIRT6 expression was consistently higher in lung cancer cell-lines than in normal lung cell-lines (Figure 12). The reinterpretation of Lu et al.'s microarray data showed that SIRT6 expression was also higher

in non-smoking lung cancer tissues than in normal lung tissue in database of Taiwan's female (Figure 13) [25]. These results indicate that cAMP signaling reduces SIRT6 protein expression in lung cancer cells.

2. cAMP signaling promoted ubiquitin-proteasome-dependent degradation of SIRT6 in H1299 cells.

To investigate how cAMP signaling reduces SIRT6 expression, the effect of GαsQL on the expression of SIRT6 mRNA level was analyzed by quantitative RT-PCR. The expression of GαsQL did not significantly change SIRT6 mRNA level (Figure 14). Then the effect of Gαs on the degradation SIRT6 was assessed. Expression of GαsQL promoted degradation of SIRT6 in the presence of cycloheximade, an inhibitor of protein synthesis in H1299 cells (Figure 15). Then, to test whether Gαs promoted SIRT6 degradation via proteasomal pathway, the effect of proteasomal inhibitor, MG-132 on SIRT6 degradation was analyzed. Treatment with MG-132 increased the SIRT6 level in both the vector- and the GαsQL-transfected cell, abolishing SIRT6-reducing effect of GαsQL completely (Figure 16). Because many proteins are

ubiquitylated before proteasomal degradation, the effect of Gas on ubiquitylation of SIRT6 was analyzed. Expression of GαsQL increased the amount of through poly-ubiquitylated SIRT6 immunoprecipitated by SIRT6 antibody in H1299 cells (Figure 17). Next, the effect of cAMP signaling activated by PGE2 or forskolin on proteasomal degradation of SIRT6 reduction was assessed. The SIRT6 reducing effect of PGE2 or forskolin was abolished by MG132 treatment (Figure 18, Figure 19). To screen the ubiquitin E3 ligase involved in SIRT6 degradation, we analyzed SIRT6 expression following knock down of several E3 ligases. Knock down of E3 ligases such as CHIP, iduna, ITCH, MDM2, and Skp2 by specific shRNA was confirmed at mRNA and protein level (Figure 20). Both the basal and the PGE2-treated expression of SIRT6 were increased by all the knock down of E3 ligases, suggesting their involvement in SIRT6 degradation (Figure 21). However, PGE2 showed the SIRT6-reducing effect in cells knock downed of MDM2 or Skp2. These results indicate that cAMP signaling promotes ubiquitylation-proteasome dependent degradation of SIRT6 in H1299 cells.

3. cAMP reduced SIRT6 expression via PKA and CREB in H1299 cells.

To probe the signaling pathway mediating the SIRT6-reducing effect of cAMP, the role of cAMP-dependent protein kinase (PKA) was assessed. Inhibition of PKA by treatment with an inhibitor (H89) or by expression of dominant negative PKA (dnPKA) abolished the SIRT6-reducing effect of Gas and PGE2 in H1299 cells (Figure 22, Figure 23). Because CREB is a well-known downstream target of PKA, involvement of CREB was examined. Inhibition of CREB activation by transfection of dominant negative CREBs (dnCREBs: CREB S133A, CREB R287L) increased the both the basal and PGE2-treated levels of SIRT6 in H1299 cells (Figure 24). Similarly, blocking binding of CREB to its targests by transfecting CRE decoy oligonucleotide increased SIRT6 expression, which was not affected by treatment with PGE2 (Figure 25). The results indicate that cAMP signaling reduces SIRT6 expression by activation of PKA and CREB pathway in lung cancer cells.

4. cAMP signaling reduced SIRT6 expression by inhibiting ERK pathway in H1299 cells.

To study the signaling pathway that mediates SIRT6-reducing effect of cAMP signaling, the effect of PGE2 treatment duration on SIRT6 expression was examined first. Treatment with PGE2 for 1 h decreased SIRT6 expression significantly, and treatment for 2 h reached a minimum expression of SIRT6 after 24 h in H1299 cells (Figure 26), which suggests that PGE2 signaling activities within 2 h after treatment is enough to decrease SIRT6 expression duration. To probe the signaling pathway, we screened the effect of mitogen activated protein kinases (MAPKs) on SIRT6 expression following treatment with specific inhibitors. Treatment with a ERK inhibitor, PD98059, decreased SIRT6 expression like PGE2 treatment, but treatment with either a JNK inhibitor (SP600125) or a p38 inhibitor (SB203580) did not change SIRT6 expression (Figure 27). Treatment with other ERK inhibitors, PD0325901 also reduced SIRT6 expression similarly (Figure 28). To confirm the effect of ERK signaling on SIRT6 expression, the ERK signaling was inhibited by expression of dominant negative ERKs. Expression of dominant negative ERK1 (dnERK1) or ERK2 (dnERK2) reduced SIRT6 expression (Figure 29). Expression of constitutively active MEK (caMEK) increased SIRT6 expression and abolished the HDAC6 reducing effect of PGE2 in H1299 cells (Figure 30).

Treatment with PGE2 decreased activating phosphorylation of c-Raf at serine-338, MEK and ERK, but increased the inhibitory phosphorylation of c-Raf at serine-259 (Figure 31). The temporal pattern of ERK phosphorylation was inversely correlated with CREB phosphorylation following PGE2 treatment (Figure 32), suggesting involvement of PKA in the phosphorylation of ERK as well as CREB. PGE2 increased inhibitory phosphorylation of c-Raf at serine-259 but decreased activating phosphorylation at serine-358. Expression of dominant negative PKA reduced the PGE2 effect on phosphorylation of both serine residues (Figure 33), which inhibited c-Raf/MEK/ERK signaling pathways and SIRT6 expression in H1299 cells. These results indicate that cAMP signaling reduces expression of SIRT6 by inhibition of Raf-MEK-ERK signaling in a PKA dependent manner in lung cancer cells.

5. Gαs augmented γ-ray-induced apoptosis by reducing SIRT6 expression in lung cancer cells.

To probe the effect of the reduced SIRT6 expression by cAMP signaling, we examined the effect on γ-ray-induced apoptosis. Activation of cAMP signaling by expression of GαsQL increased the

cleavage of caspase-3 and PARP, and Annexin V stained in the γ-ray irradiated H1299 cells (Figure 34, Figure 35). Coexpression of wild type SIRT6 abolished the effect of GαsQL on the cleavage of caspase-3 and PARP and Annexin V staining. SIRT6 expression alone decreased γ-ray-induced apoptosis, and expression of a dominant negative SIRT6 also decreased γ-ray-induced apoptosis less efficiently compared with wild type SIRT6 (Figure 36). Activation of cAMP signaling by PGE2 treatment also increased the cleavage of caspase-3 and PARP, which was also blocked by expression of wild SIRT6 (Figure 37). The expression of Gαs increased γ-ray-induced cleavage of casapse-3 and PARP in A549 cells similar to H1299 cells (Figure 38). These results suggest that cAMP signaling reduces SIRT6 expression, which mediates augmentation of γ-ray-induced apoptosis by cAMP signaling in lung cancer cells.

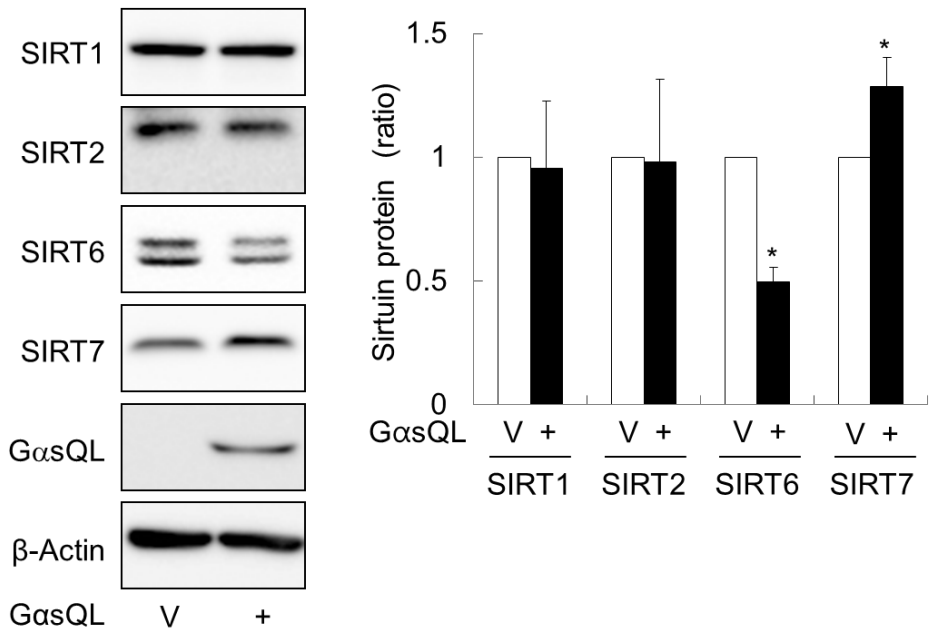

Figure 8. Effects of Gαs on the expression of sirtuin isoforms in H1299 human lung cancer cells.

H1299 cells were transfected with EE-tagged GαsQL (GαsQL), or vector pcDNA3.1 (V) by calcium phosphate method and incubated for 24 h. The cell were harvested and homogenized, and analyzed by western blotting. SIRT3, 4, and 5 proteins were not detected because of mitochondria protein.

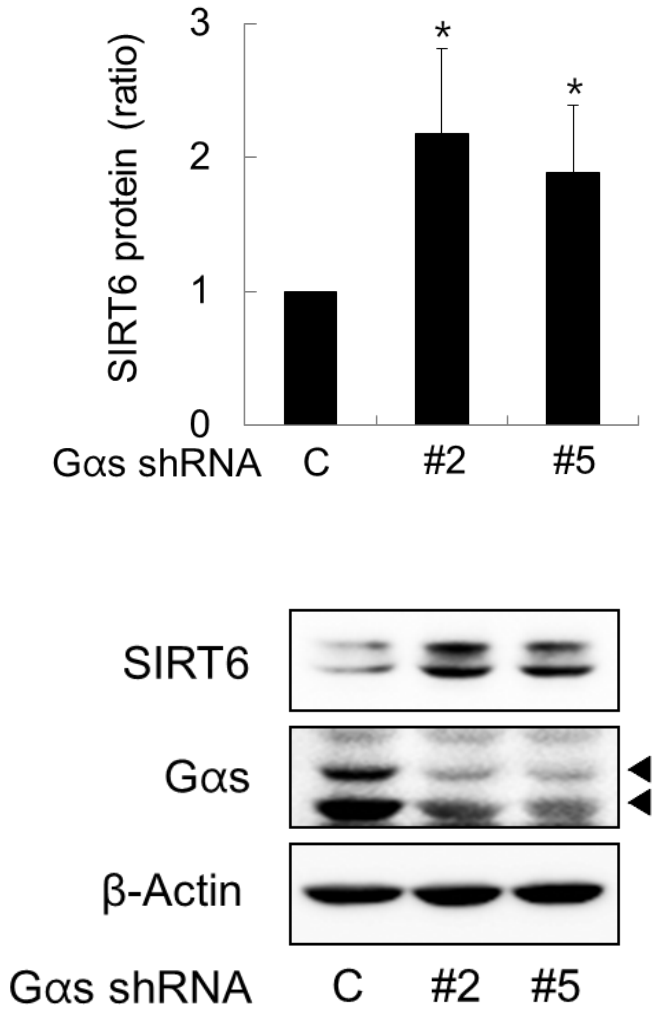

Figure 9. Effects of Gαs knock down on the expression of SIRT6.

The expression of Gαs was knock downed by transfecting 10 μg sh RNA, and shRNA containing scrambled RNA was used as the control (C). After 48 h, the cell were harvested, and homogenized, and analyzed by western blotting.

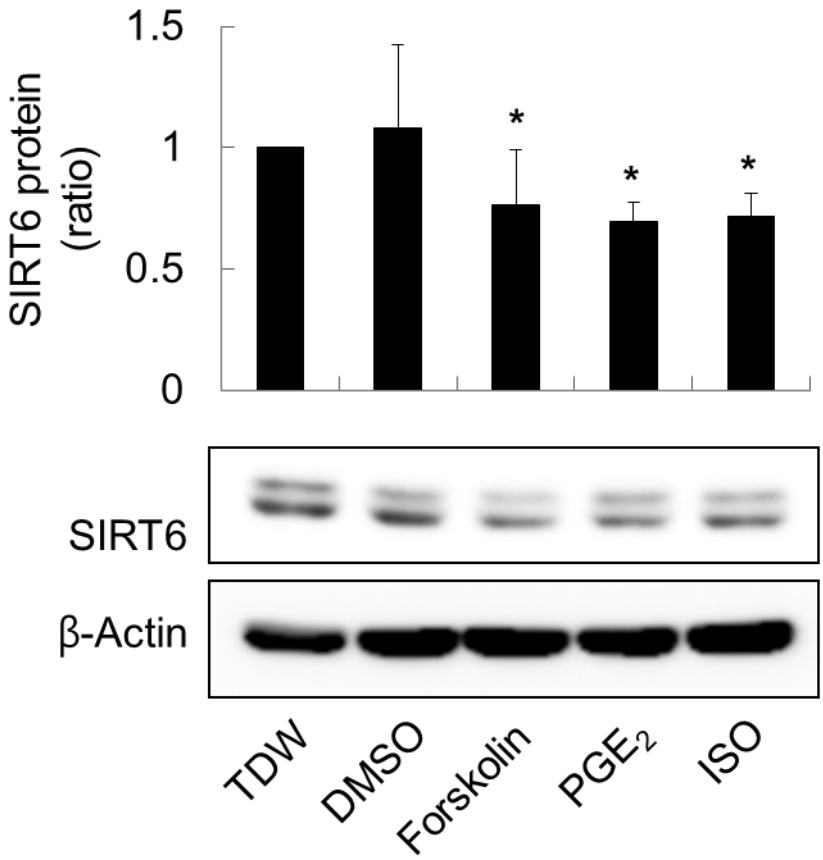

Figure 10. Effects of cAMP signaling on the expression of SIRT6

H1299 cells were treated with 40 μM forskolin, 10 μM prostaglandin E2 (PGE2), 1 μM isoproterenol (ISO), DMSO, or TDW (3rd distilled water) as vehicle for 24 h before western blot analysis.

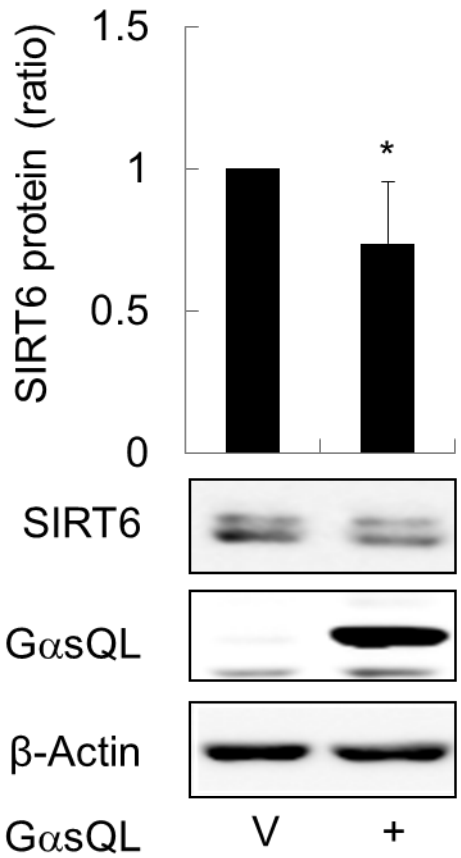

Figure 11. Effects of Gαs on the expression of SIRT6 in A549 human lung cancer cells

A549 cells were transfected with EE-tagged GαsQL (GαsQL), or vector pcDNA3.1 (V) by calcium phosphate method and incubated for 24 h. The cells were harvested, and analyzed by western blot.

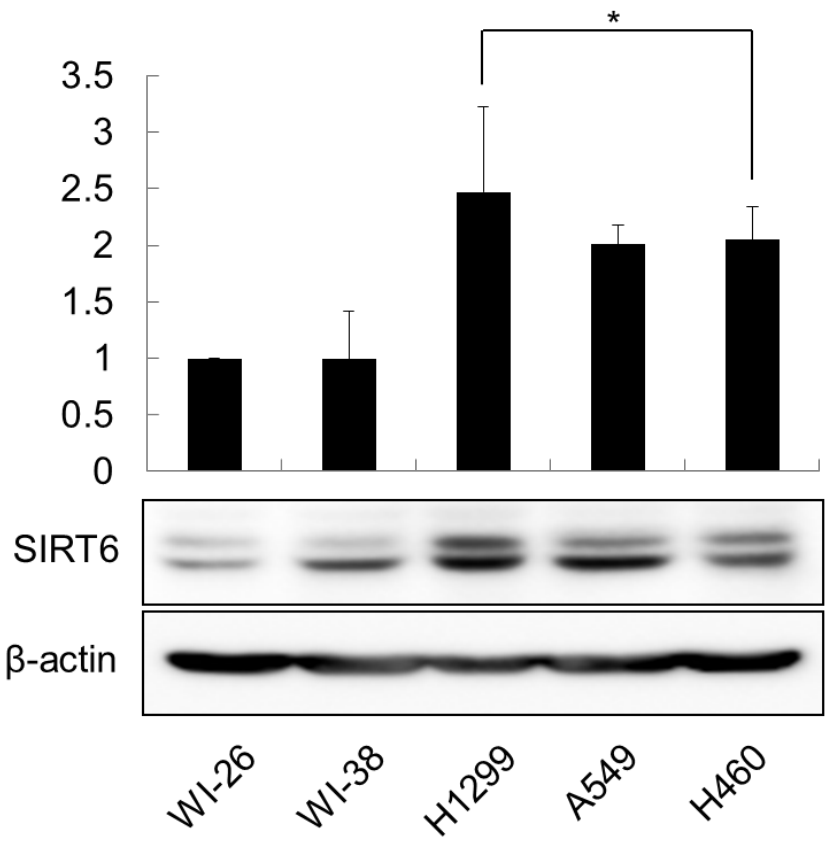

Figure 12. To confirm the SIRT6 expression of variable normal lung cells, or lung cancer cells.

Normal lung cells (Wi-26, and Wi-38), and lung cancer cells (H1299, A549, and H460) were passaged, and incubated during 24 h. The cells harvested and homogenized, and analyzed by western blotting. An asterisk indicates a statistically difference compare with respective control or vector-transfected cells ($p<0.05$, Mann-Whitney U test).

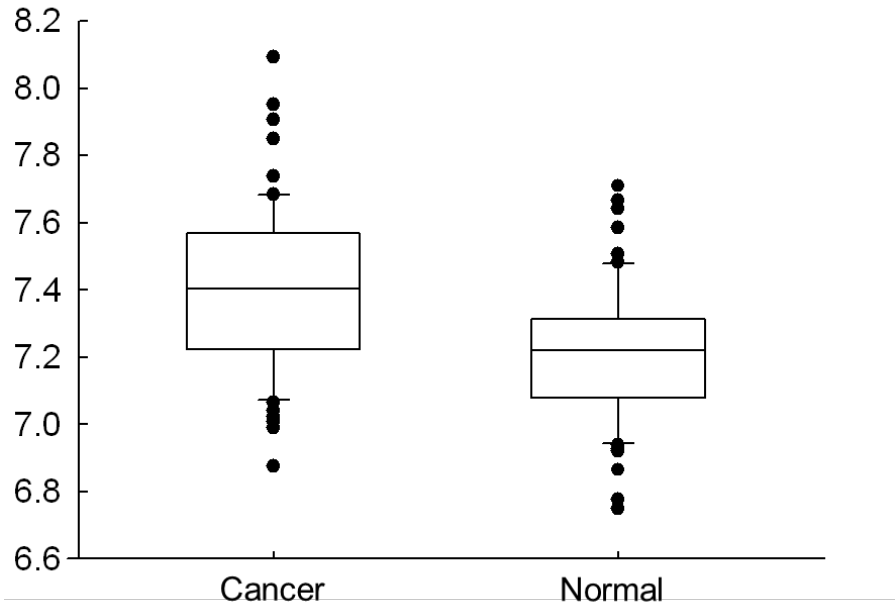

Figure 13. Overexpression of SIRT6 in non-smoking female lung cancer or normal tissues in Taiwan

Microarray expression of the lung cancer database was downloaded from NCBI database (GDS3837) to examine the absolute expression value of SIRT6 between cancer (lung cancer, n=60), and normal (normal lung, n=60) tissues.

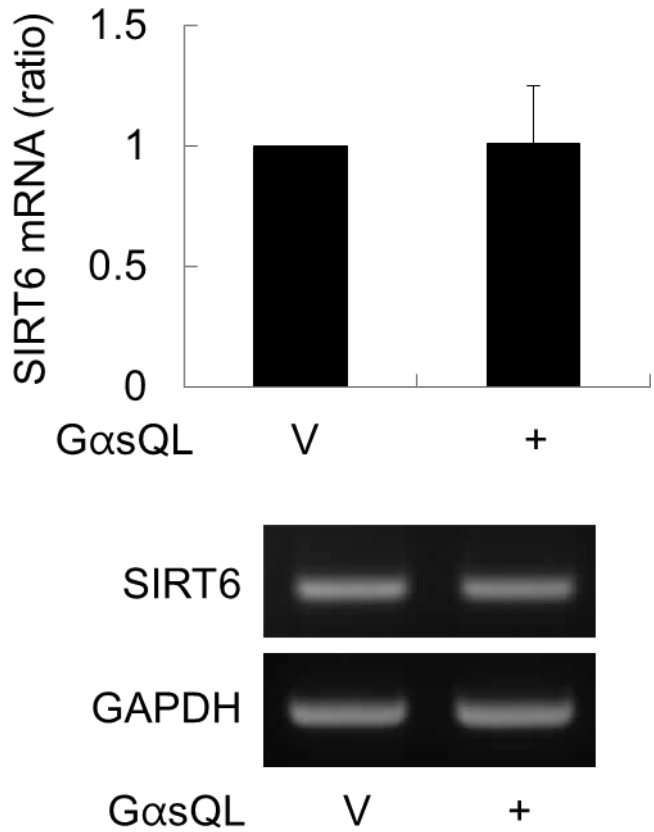

Figure 14. Effects of Gαs on the expression of SIRT6 mRNA.

The expression of SIRT6 mRNA was analyzed by RT-PCR, and real-time quantitative PCR 24 h after transfection of GαsQL in H1299 cells. SIRT6 mRNA level was analyzed on the basis of GAPDH control by qPCR.

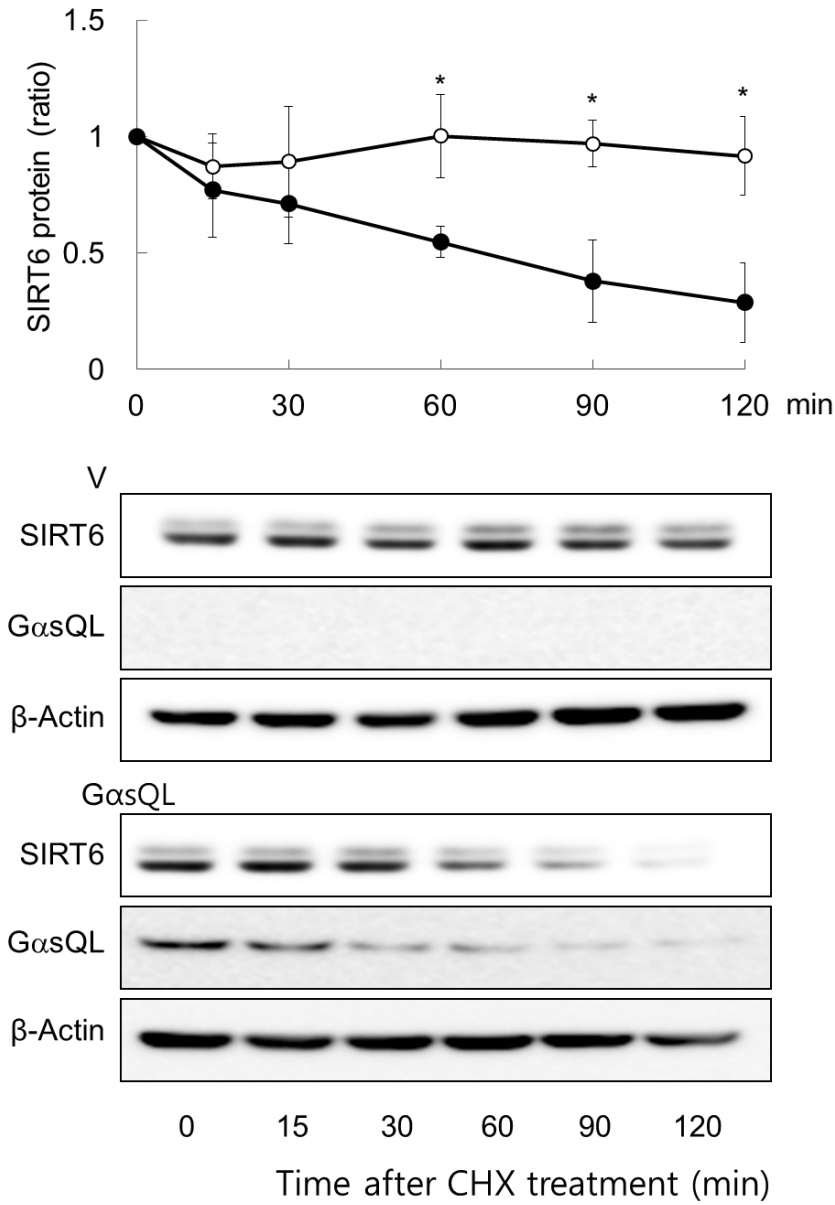

Figure 15. Effects of Gαs on the degradation of SIRT6 protein.

H1299 cells were transfected with GαsQL or vector (V), were treated with 50 μg/ml cycloheximide (CHX) 24 h after transfection, and harvested at the indicated times.

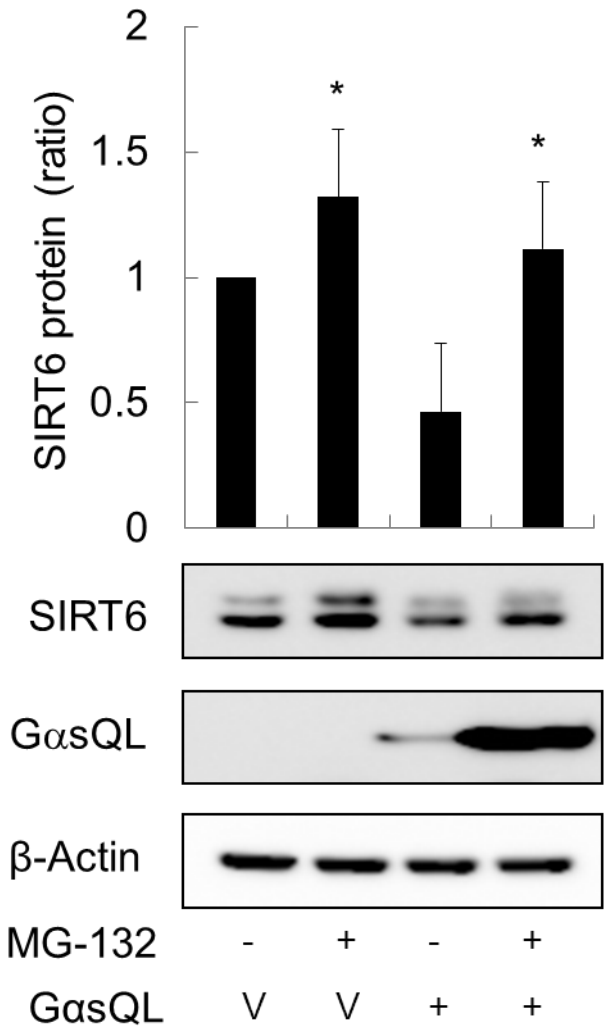

Figure 16. Effects of MG-132 treatment on the SIRT6 expression.

H1299 cells were transfected GαsQL, or vector (V)and incubated 6 h. Then, the cells were treated with 10 μM MG-132 for 18 h before harvest for analysis.

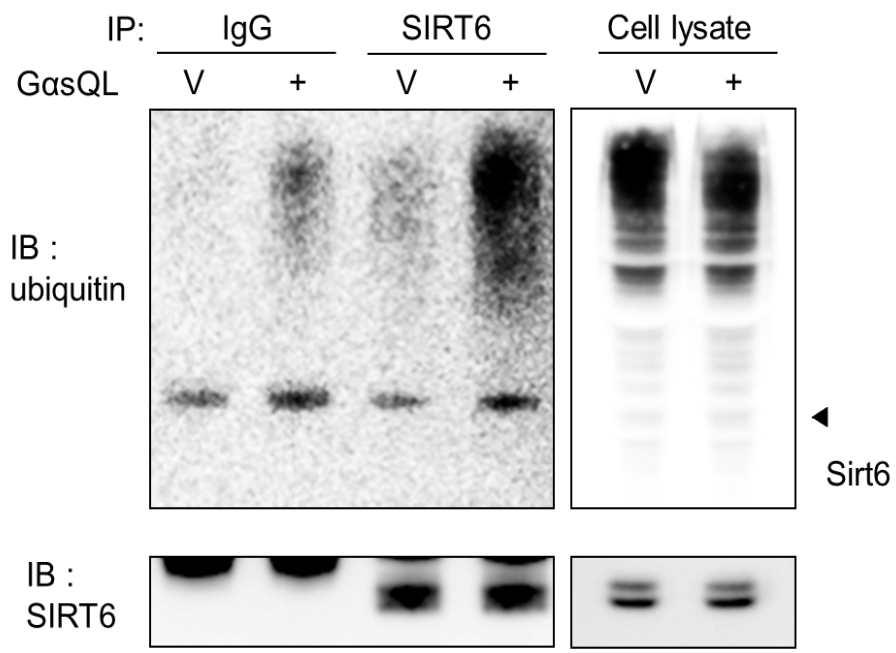

Figure 17. Effects of Gαs on the ubiquitylation of SIRT6.

H1299 cells were transfected with GαsQL or vector (V) and incubated 24 h before harvest. The whole lysate (800 μg) was precleared by Protein A agarose for 1 h, obtained supernatant sample. The precleared lysate was incubated with anti-SIRT6 antibody (1 μg), or control IgG at 4℃ for 16 h, and the with a Protein A agarose for 2 h. The final samples were washed 3 times and analyzed by western blotting.

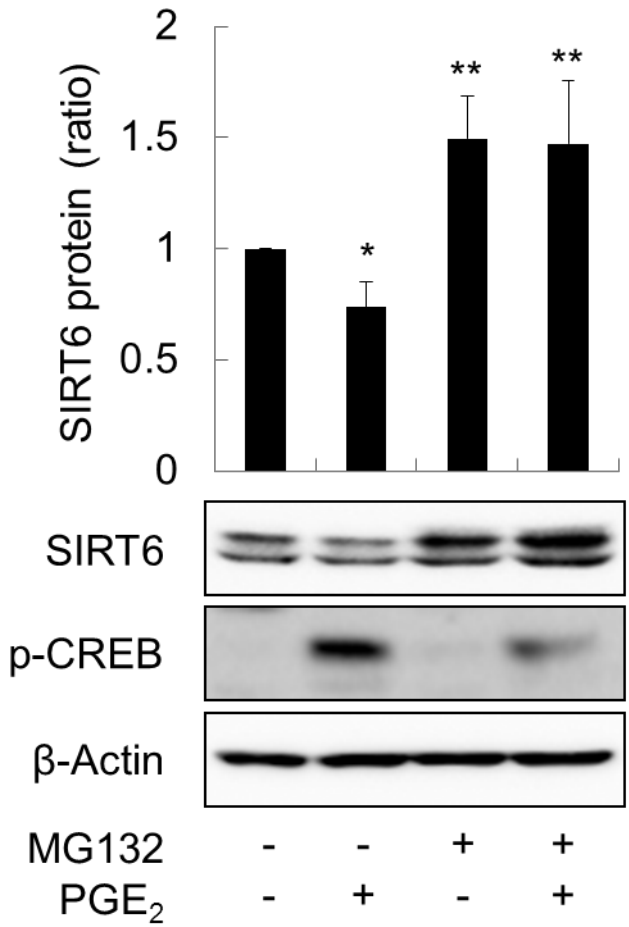

Figure 18. Effects of PGE2 on the degradation of SIRT6 protein.

H1299 cells were treated with 10 μM in the presence/absence of 10 μM MG-132 or DMSO for 24 h before western blotting analysis.

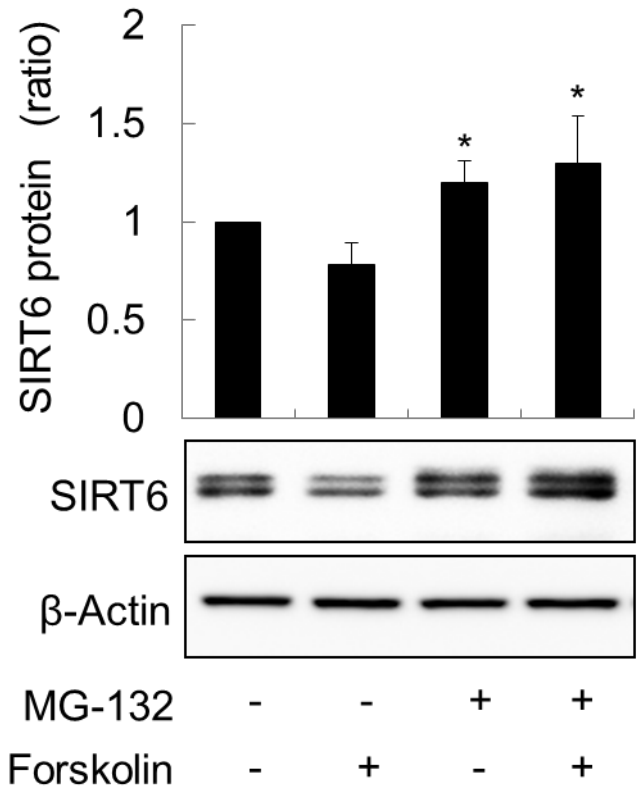

Figure 19. Effects of forskolin on the degradation of SIRT6 protein.

H1299 cells were treated with 40 μM forskolin in the presence/absence of 10 μM MG-132, or DMSO for 24 h before western blotting.

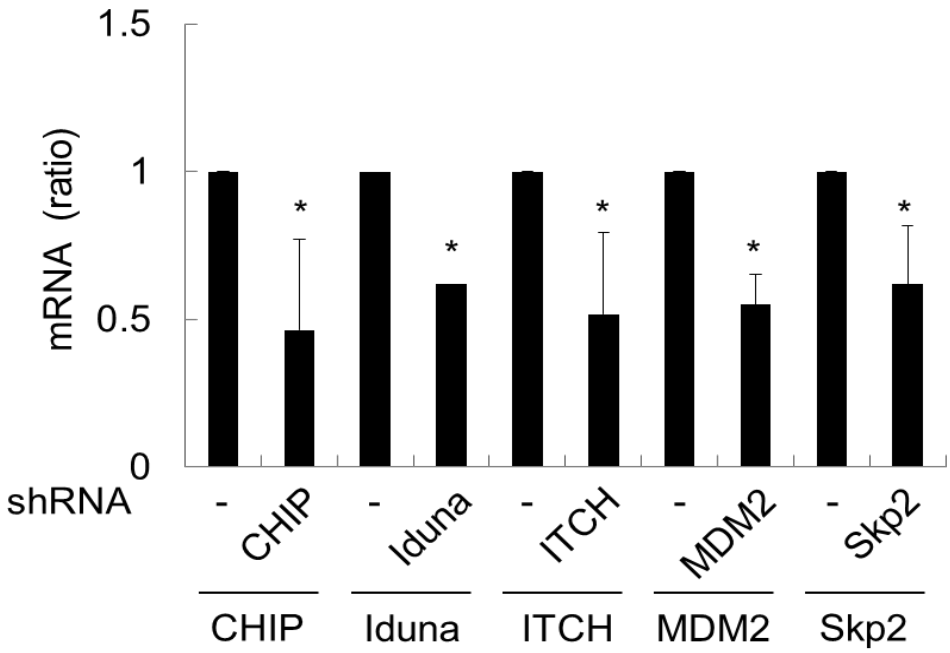

Figure 20. To confirm expression of E3 ligases mRNA in H1299 cells

H1299 cells were transfected with shRNA against CHIP, iduna, Mdm2, Skp2, or scrambled shRNA as a control. After 48 h, the cells were harvested, and analyzed by qPCR.

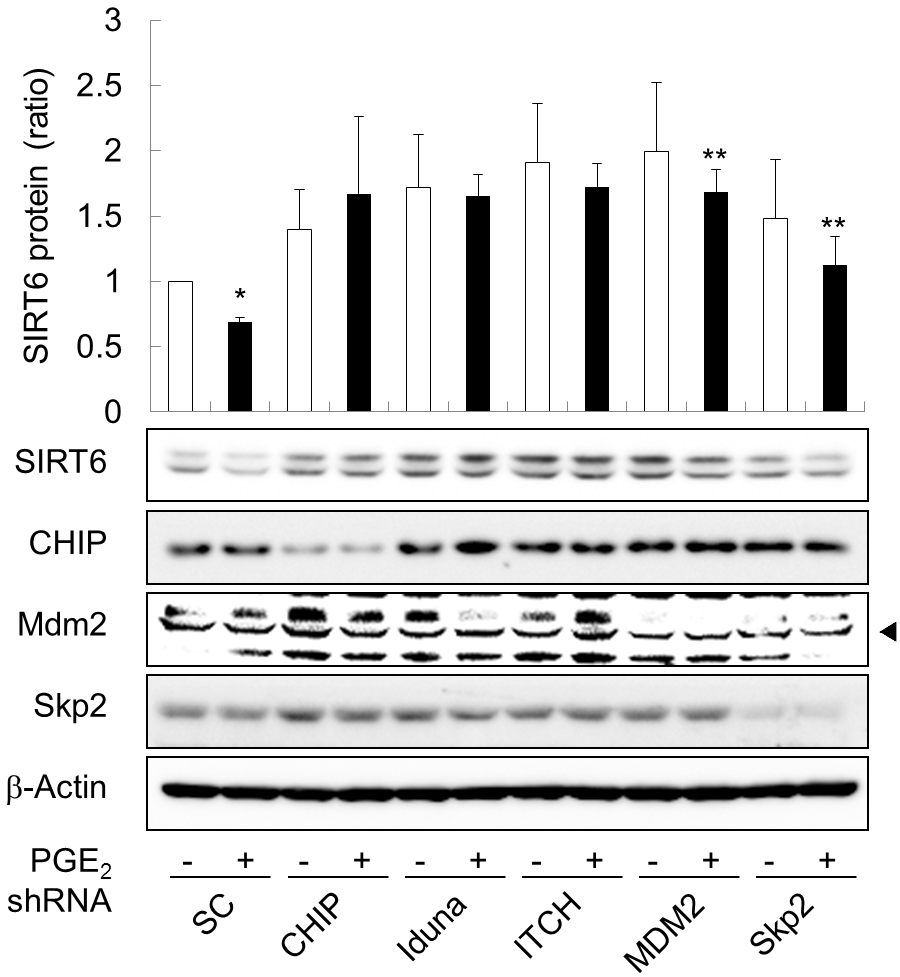

Figure 21. Effects of knock down of E3 ligases on PGE2-promoted degradation of SIRT6 protein.

H1200 cells were transfected with shRNA against CHIP, iduna, ITCH, Mdm2, Skp2, or scrambled shRNA as a control. After incubation for 24 h, the transfected cells were treated with 10 μM PGE2 for 24 h before western blot analysis.

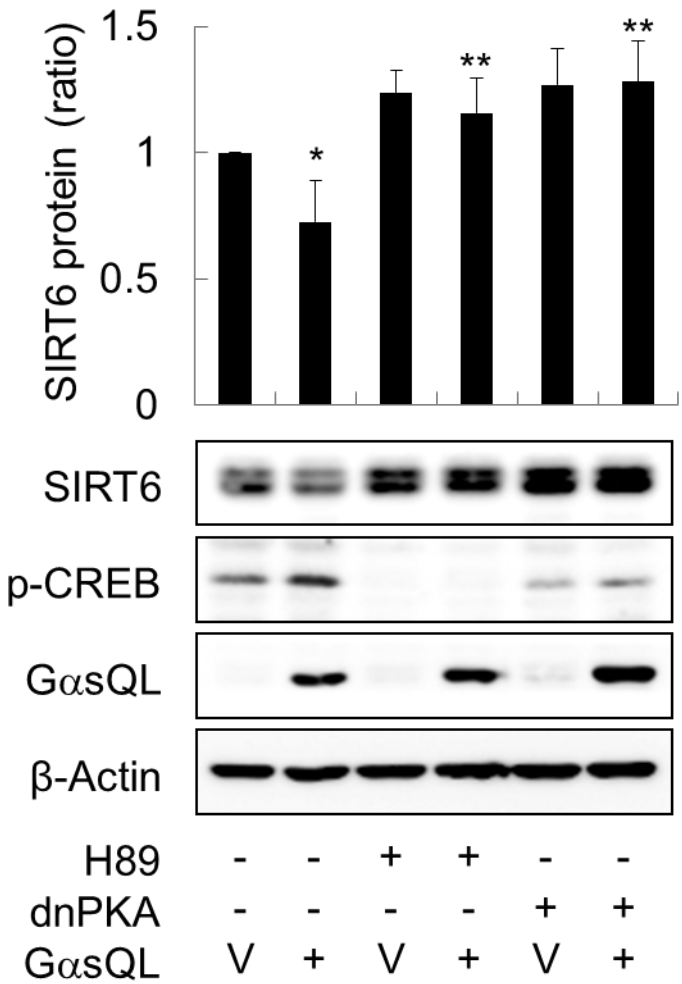

Figure 22. Inhibition of PKA blocked the GαsQL effect on SIRT6 expression.

H1299 cells were transfected with GαsQL, a dominant negative PKA (dnPKA), or vector (V), and incubated for 24 h. Then, the cells were treated with 20 μM H89, or DMSO for 24 h before western analysis.

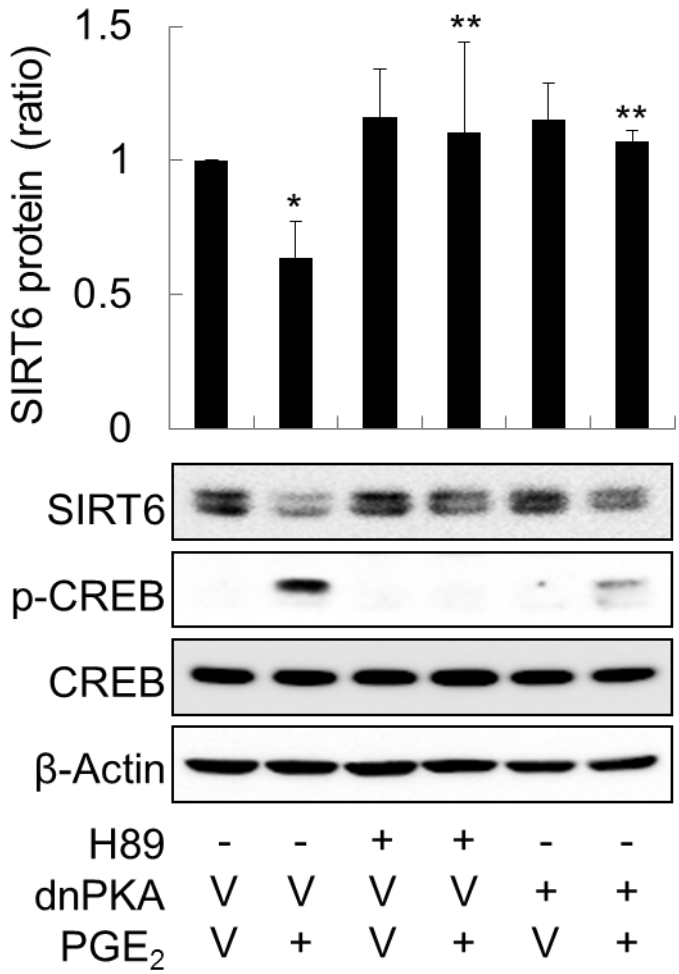

Figure 23. Inhibition of PKA blocked the PGE2 effect on SIRT6 expression.

H1299 cells were transfected with dnPKA, or control vector (V), and incubated for 24 h. Then, the cells were treated with 20 μM, 10 μM PGE2, or DMSO for 24 h before western blotting.

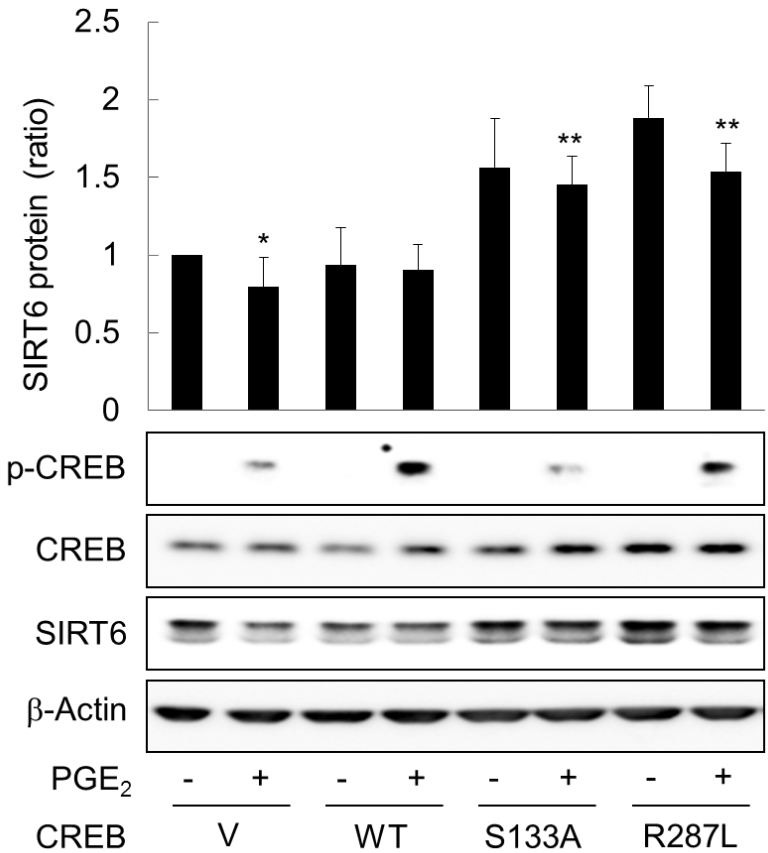

Figure 24. Inhibition of CREB activation increased SIRT6 expression.

H1299 cells were transfected wild type CREB (WT), dominant negative CREBs (S133A, or R287L), or control vector (V), and incubated for 24 h. Then, the cells were treated with 10 μM PGE2, or DMSO for 24 h before western blotting analysis. The phosphorylation of CREB (p-CREB) was analyzed 30 min after PGE2 treatment.

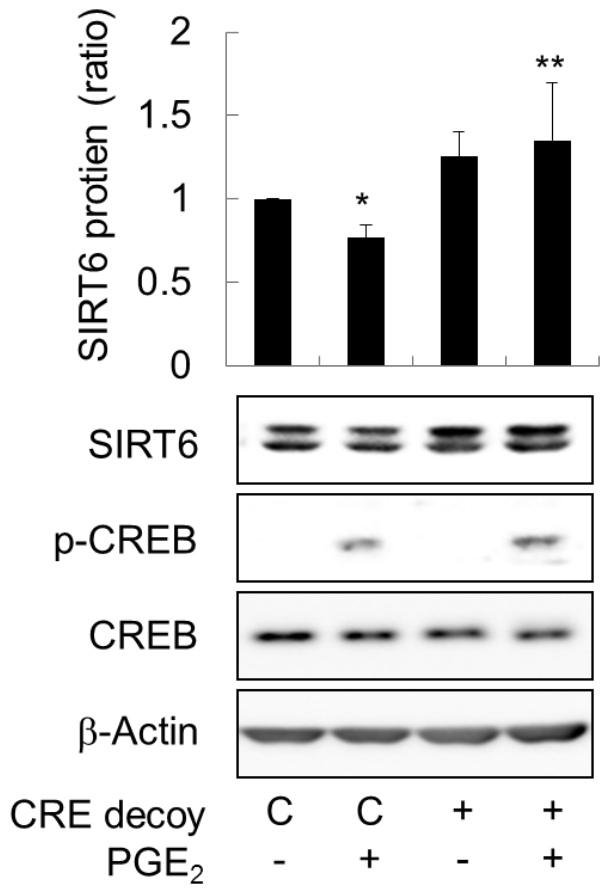

Figure 25. Inhibition of CREB binding to CRE increased SIRT6 expression.

H1299 cells were transfected with oligonucleotides (CRE decoy, or CRE control), and incubated for 24 h. Then, the cells were treated with 10 μM, or DMSO for 24 h before western analysis. The phosphorylation of CREB (p-CREB) was analyzed 30 min after PGE2 treatment.

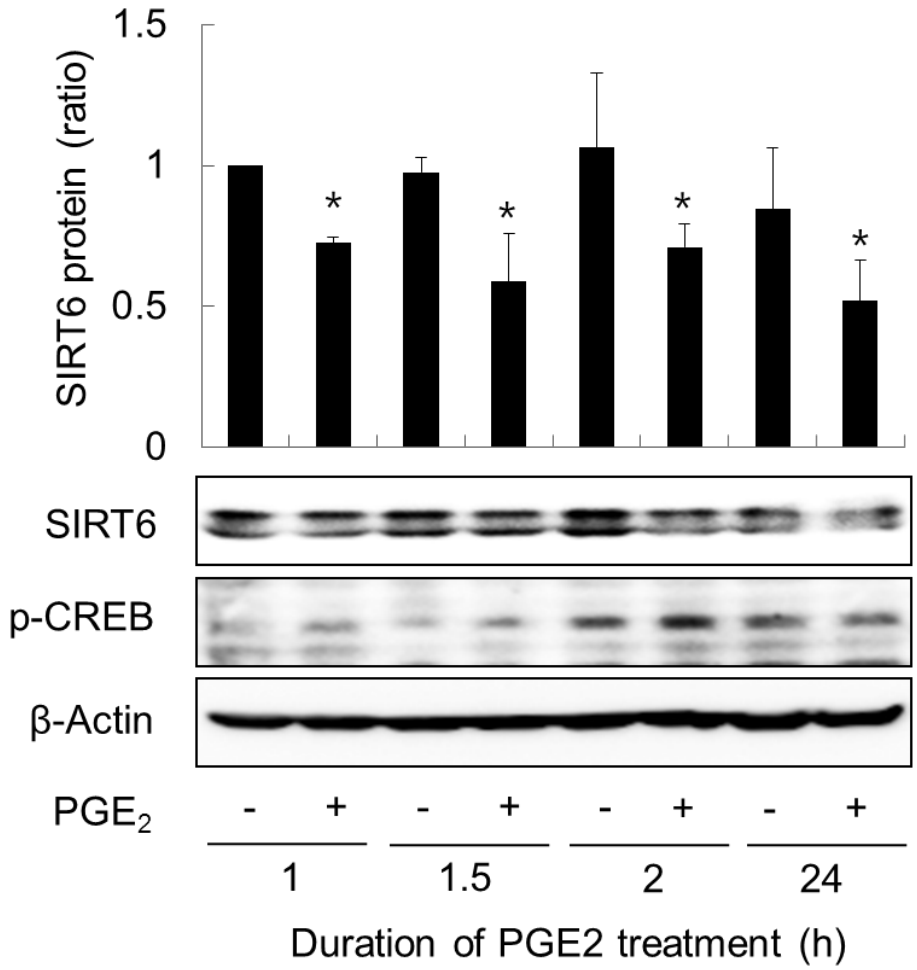

Figure 26. Effects of duration of PGE2 treatment on the expression of SIRT6 protein.

H1299 cells were treated with 10 μM PGE2, or DMSO until indicated time. After indicated time, the cells were changed fresh media, and incubated remaining time until 24 h. Then, the cells were harvested, and analyzed by western blotting.

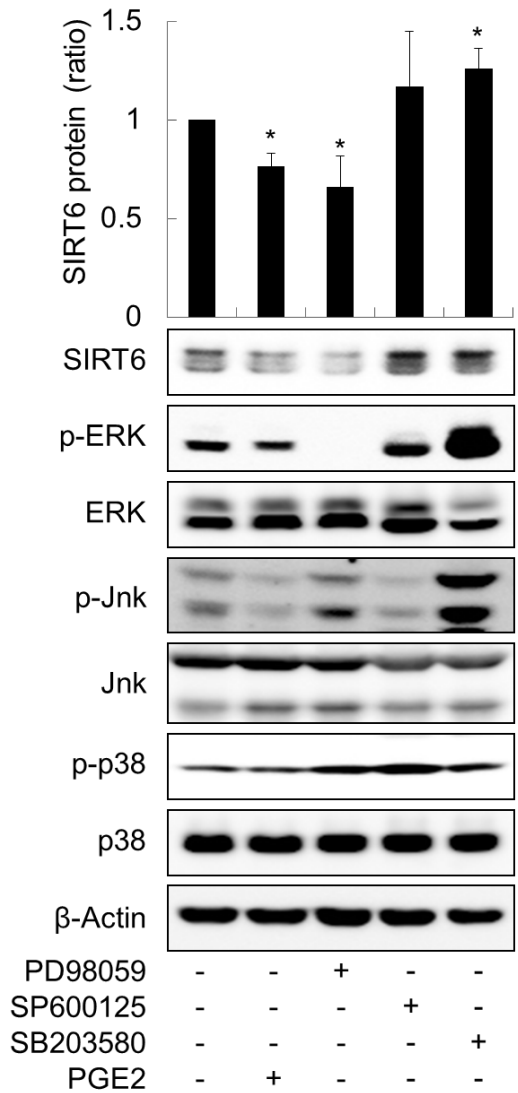

Figure 27. Effects of MAPK inhibition on SIRT6 expression.

H1299 cells were treated with 10 μM PGE2, 40 μM PD98059 (Erk inhibitor), 20 μM SP600125 (JNK inhibitor), 20 μM SB203580 (p38 inhibitor), or DMSO for 2 h, and harvested and analyzed by western blotting. The expression of SIRT6 was detected 24 h after treatment.

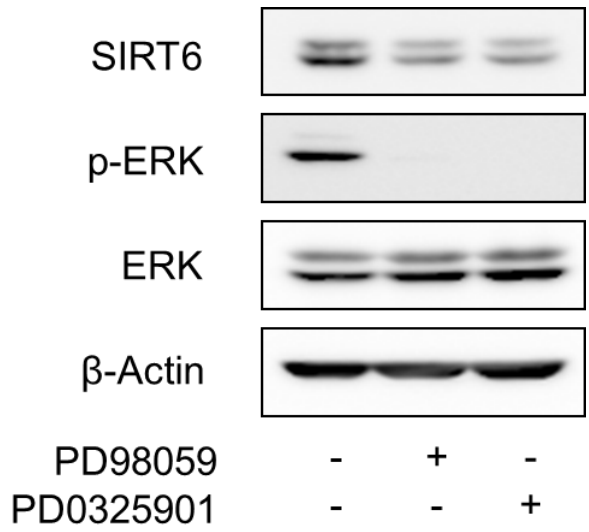

Figure 28. Effects of Erk inhibition on SIRT6 expression

H1299 cells were treated with 40 μM PD98059, 10 μM PD0325901, or DMSO for 24 h, and harvested and analyzed by western blotting.

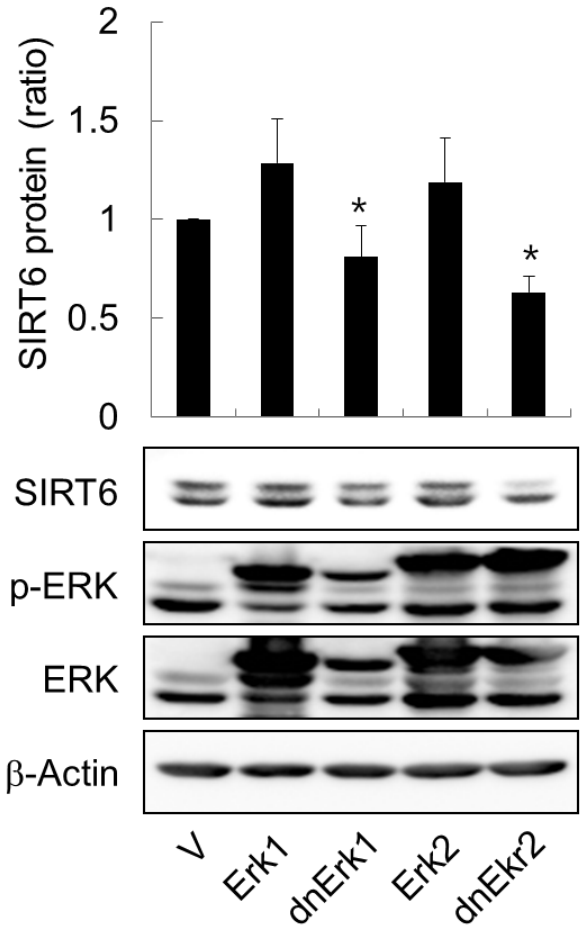

Figure 29. Effects of Erk inhibition with dominant negative ERKs (dnErks) on SIRT6 expression.

H1299 cells were transfected with ERK1, ERK2, dominant negative ERKs (dnERK1, dnERK2), or control vector (V) and incubated 24 h. The cells were harvested, and analyzed by western blotting.

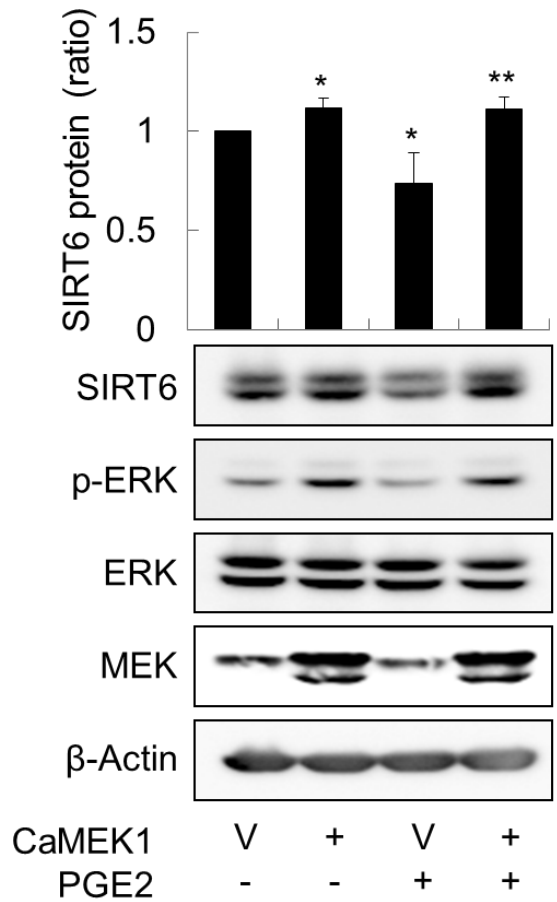

Figure 30. Effect of ERK activation on PGE2-induced inhibition of SIRT6 expression.

H1299 cells were transfected with constitutively active MEK1 (caMEK1), or control vector (V), and incubated 24 h. The transfected cells were treated with 10 μM PGE2 for 30 min before western blot analysis. The expression of SIRT6 was analyzed 24 h after PGE2 treatment.

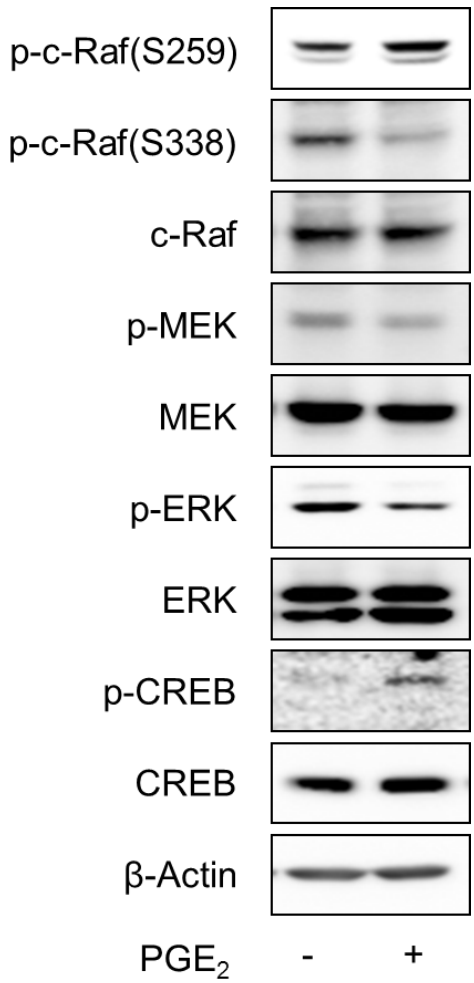

Figure 31. Effects of PGE2 treatment on the activity of Raf, Mek, and Erk.

H1299 cells were treated with 10 μM PGE2, and incubated for 30 min. After treatment, the cells were harvested, and analyzed by western blotting.

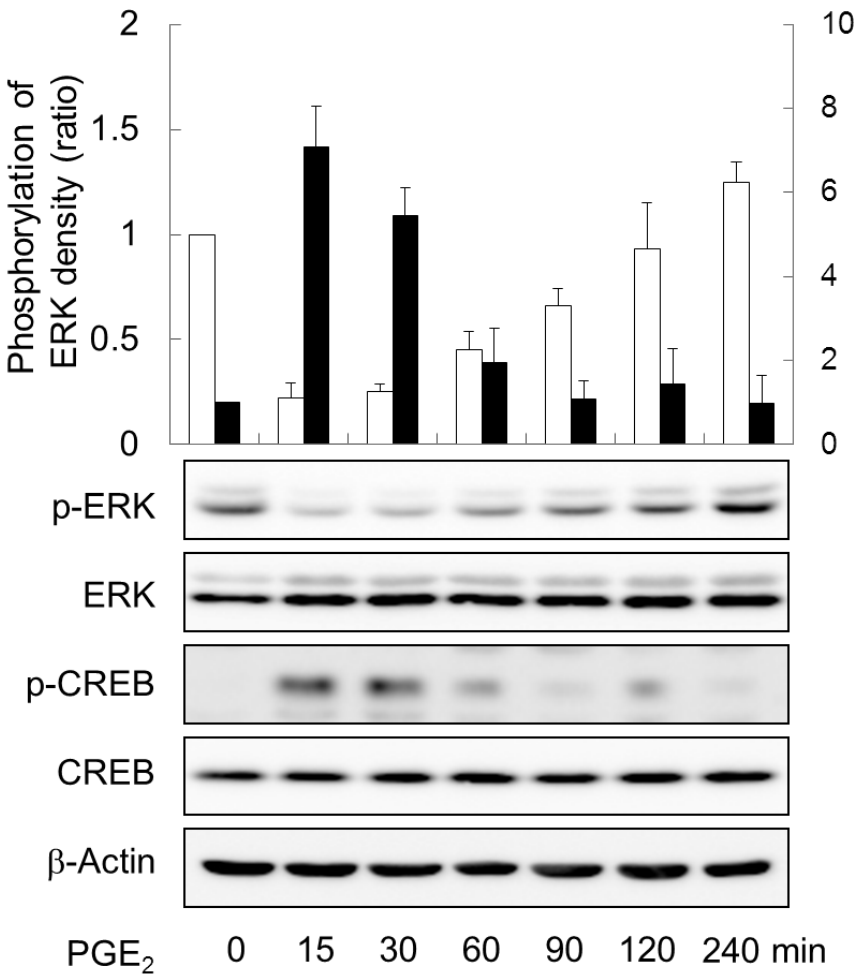

Figure 32. Effects of PGE2 treatment on phosphorylation of Erk, and CREB until 240 min.

H1299 cells were treated with 10 μM PGE2 for each time (15, 30, 60, 90, 120, and 240 min). The cells were harvested at each time, and analyzed western blotting.

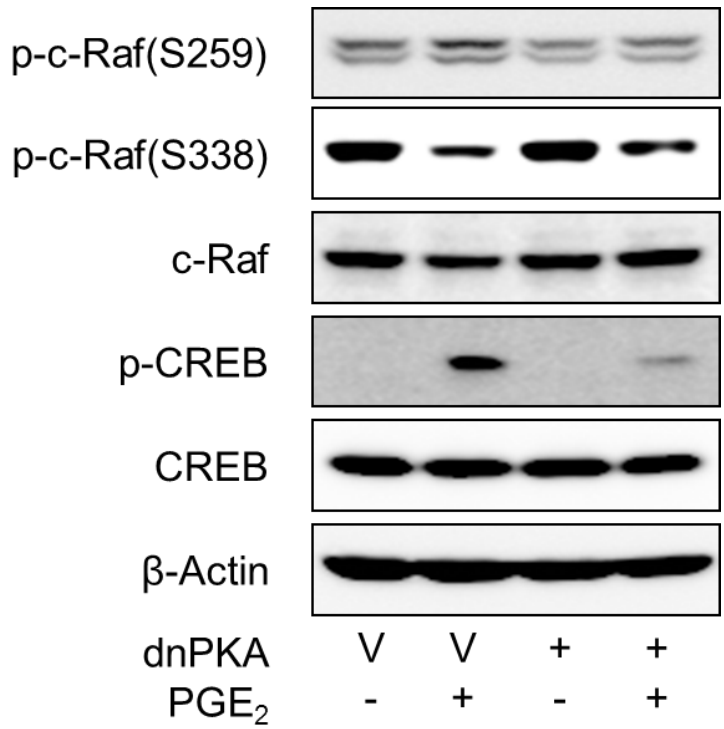

Figure 33. Effect of PGE2 on the phosphorylation of c-Raf.

H1299 cells were transfected with dnPKA, or control vector (V), and incubated 24 h. The transfected cells were treated with 10 μM PGE2 for 30 min before western blotting.

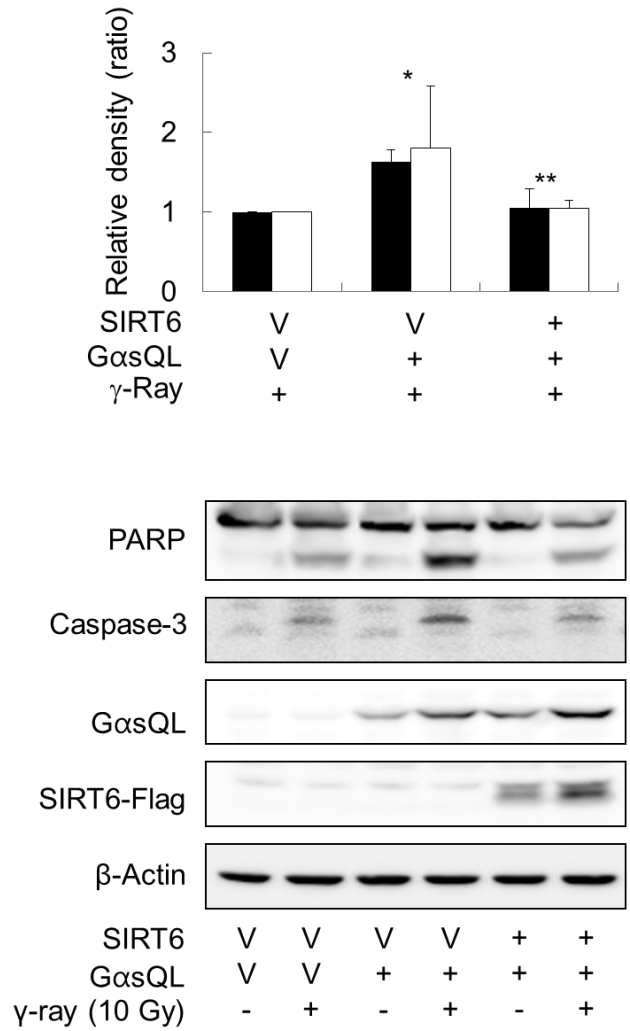

Figure 34. Effects of Gαs and SIRT6 on γ-ray-induced cleavage of caspase-3, and PARP.

H1299 cells were transfected GαsQL, wild-type SIRT6 (wtSIRT6), or control vector (V) followed by incubation for 24 h. The cells were irradiated with γ-rays (10 Gy), and incubated 24 h. Then, the cells were harvested, and analyzed by western blot.

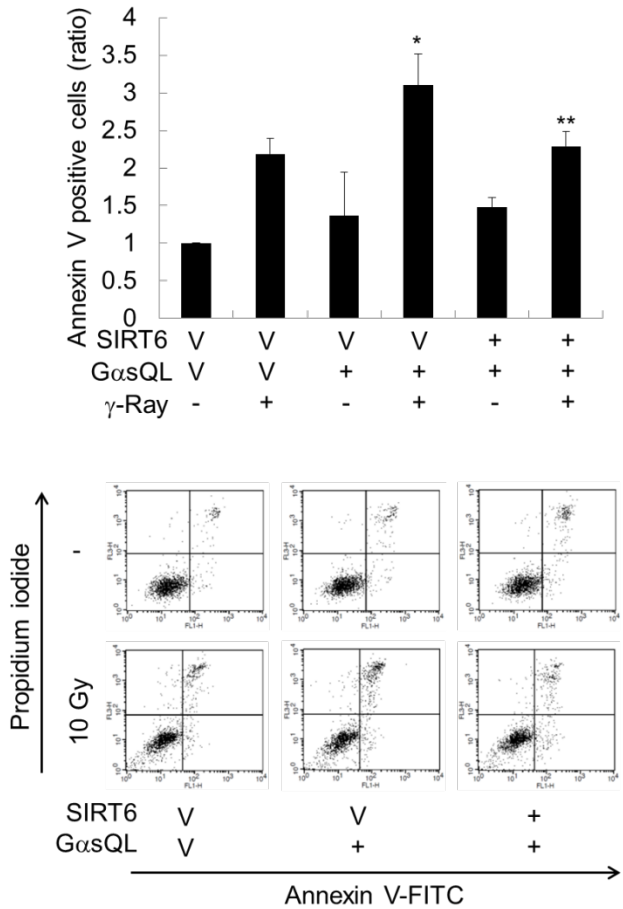

Figure 35. Effects of Gαs and SIRT6 on γ-ray-induced Annexin V-staining.

H1299 cells were transfected GαsQL, wtSIRT6, or control vector (V) followed by incubation for 24 h. The cells were irradiated with γ-rays (10 Gy). Then, the cells were incubated for 24 h before analysis of apoptosis by flow cytometry after staining with Annexin V and propidium iodide (PI). The bar graph presents the ratio of Annexin V positive cells in the whole cells.

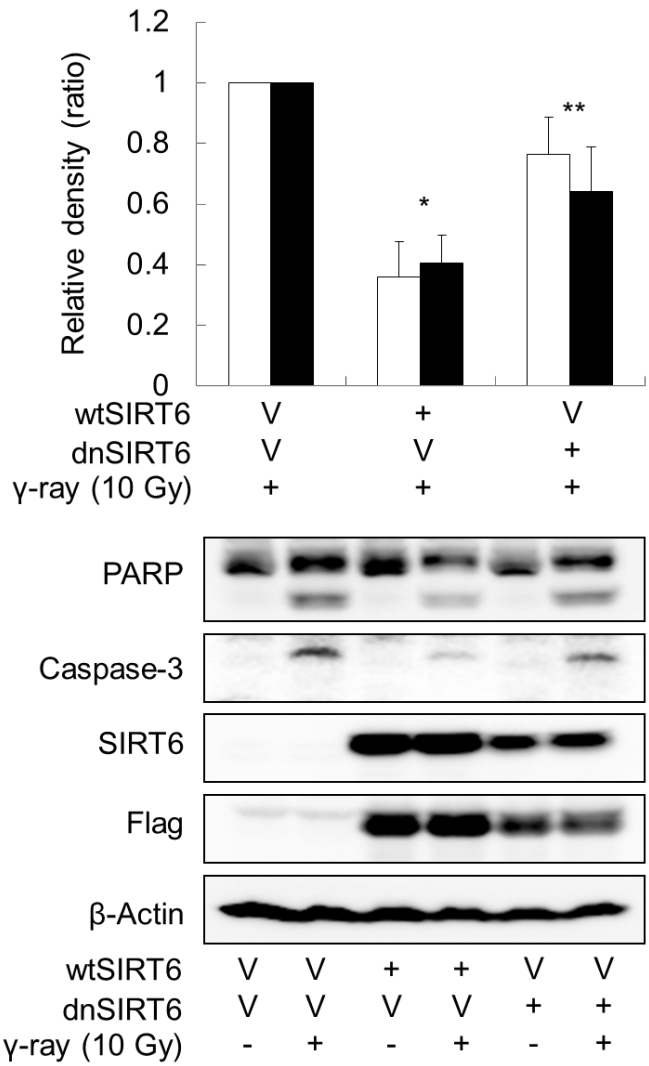

Figure 36. Effects of SIRT6 on γ-ray-induced apoptosis

H1299 cells were transfected with wtSIRT6, dominant negative SIRT6 (dnSIRT6), or control vector (V), and incubated for 24 h. The cells were irradiated with γ-rays (10 Gy) followed by incubation for 24 h. Then, the cells were harvested, and analyzed of apoptosis by western blotting.

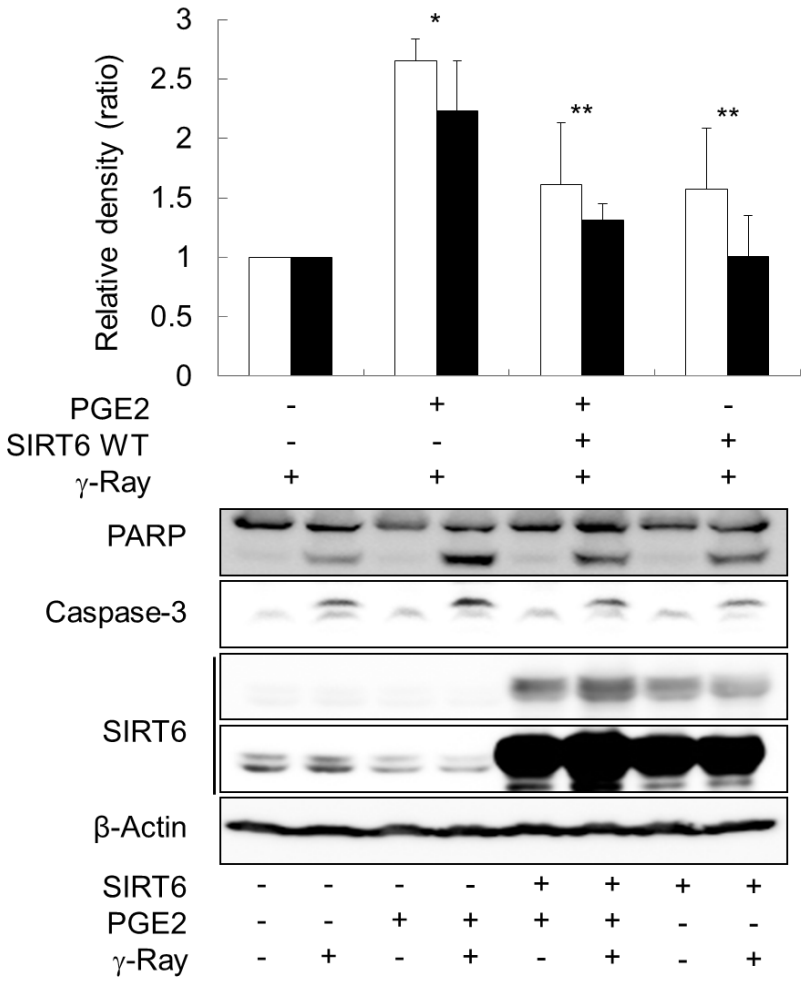

Figure 37. Effect of PGE2 on the γ-ray-induced apoptosis in H1299 cells.

H1299 cells were transfected wtSIRT6, or vector (V) followed by incubation for 24 h. The cells were irradiated with γ-rays (10 Gy) in the presence or absence of pretreatment of with PGE2 for 30 min. Then, the cells were incubated for 24 h before analysis of apoptosis by western blotting.

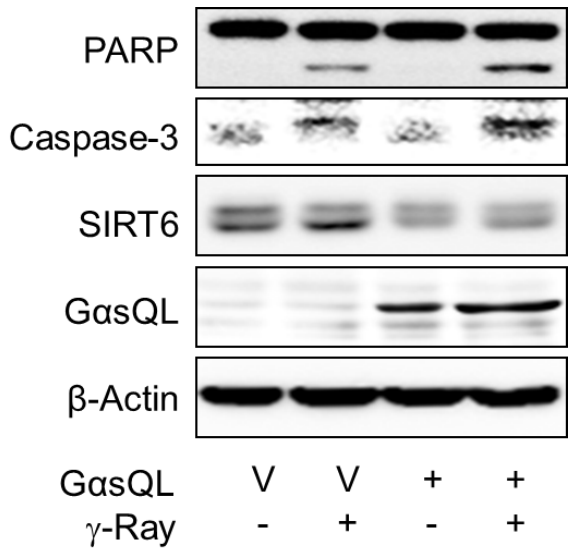

Figure 38. Effects of Gαs on γ-ray-induced apoptosis in A549 cells.

A549 cells were transfected with GαsQL, or control vector (V), and incubated for 24 h. The cells were irradiated with γ-rays (10 Gy), and incubated for 24 h. Then, the cells were harvested, and analyzed by western blot.

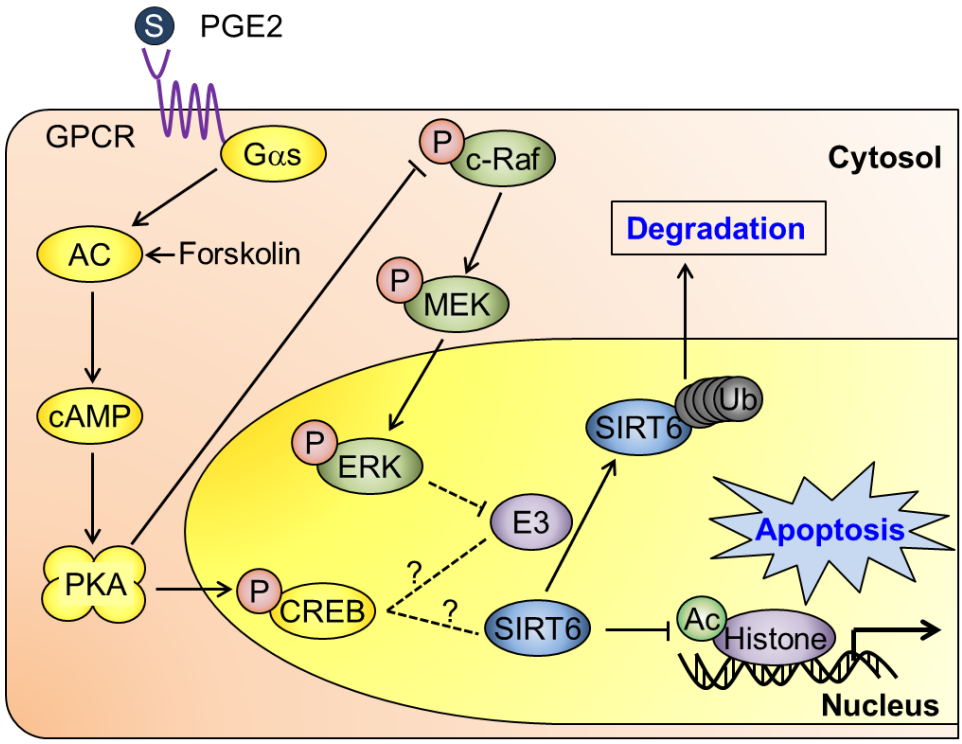

Figure 39. A proposed model for cAMP signaling reduces SIRT6 expression in lung cancer cells.

Discussion

This study was performed to investigate the effect of cAMP signaling on SIRT6 expression, its molecular mechanisms, and functional significance in lung cancer cells. We found that cAMP signaling reduces SIRT6 expression by promoting ubiquitin-proteasomal degradation, that the promotion of Sirt6 degradation is mediated by PKA-dependent inhibition of Raf-Mek-Erk pathway, and that the reduced SIRT6 expression contributes to the augmentation of γ-ray induced apoptosis of non-small cell lung cancer cells.

Our finding that cAMP signaling reduces SIRT6 expression in lung cancer cells is supported by the results that SIRT6 protein expression is reduced following the activation of cAMP signaling system by expression of constitutively active Gαs that stimulates adenylate cyclases, treatment with Gαs-coupled receptor agonists such as prostaglandin E2 (PGE2) and isoproterenol, or treatment with an adenylate cylase activator, forskolin, in H1299 human non-small cell lung cancer lines. The cAMP signaling also reduced

SIRT6 expression in A549 cells, another human lung cancer cells, suggesting the effect is not limited to a specific lung cancer cell line. The cAMP signaling did not affect the expression of SIRT isoforms other than SIRT6, implying an SIRT6 isoform-specific effect.

The SIRT6 expression level was reported to increase in cultured cells upon nutrient deprivation, in rodents following fasting or a calorie-restricted diet [48], and cerebral ischemia decreased SIRT6 expression [49]. Several molecules such as microRNAs, and peroxisome proliferator-activated receptor gamma have been reported to regulate Sirt6 expression [50] [51]. cAMP signaling cAMP activated by leptin triggers catecholamine stimulates HDAC4 activity through the PKA-dependent manner to reduce inflammatory gene expression [52], and CREB was reported to induce SIRT1 expression upon low nutrient availability [53]. However, the effect of cAMP signaling on SIRT6 expression has not been known yet, and in this paper, we present a novel mechanism that cAMP signaling decreases SIRT6 expression in lung cancer cells.

This study shows that the cAMP signaling reduces SIRT6 expression by promoting ubiquitin-proteasomal degradation of

SIRT6. This finding is supported by the results that cAMP signaling did not increase SIRT6 mRNA levels, but stimulated degradation of SIRT6 after inhibition of protein biosynthesis, that inhibition of proteasome abolished the degradation-stimulating effect of cAMP signaling, and that cAMP signaling increased ubiquitylation of SIRT6. We also suggest that several ubiquitin E3 ligases such as MDM2, CHIP, iduna, ITCH, and Skp2 were involved in the degradation SIRT6 from the result that knock down of the enzyme caused an increase in SIRT6 expression. However identification of the E3 ligase that mediates cAMP effect needs further investigation. Regulation of SIRT6 expression by ubiquitin-proteasome dependent degradation has been indicated by several recent reports. SIRT6 was found to be phosphorylated by AKT, ubiquitylated by MDM2, and then degraded by proteasome in breast cancer cells [54]. The ubiquitin ligase CHIP is involved in protein quality control and regulation of aging and life span in mice. CHIP was shown to ubiquitylate noncanonically SIRT6 at lysine 170, which prevents SIRT6 canonical ubiquitylation by other ubiquitin ligases [55]. USP10 was reported to interact with, deubiquitylate, and stabilize SIRT6 in colon cancer cells [56]. cAMP regulates ubiquitin-proteasomal degradation of several proteins by controling ubiquitin

E3-lgases such as including atrogin-1 in skeletal muscle [57], NEDD4L [58], and SCF-type ubiquitin E3 ligase [59]. Our result that knock down of MDM2 with siRNA increased basal SIRT6 expression in H1299 lung canacer cells is compatible with the Thirumurthi et al's report, suggesting regulation of SIRT6 ubiquitylation by MDM2 [54], but our result that treatment with PGE2 still reduced the SIRT6 expression in MDM2 knock downed cells suggests that cAMP signaling induces ubiquitylation in MDM2-independent pathway. This study showed that knock down of CHIP by siRNA increased the SIRT6 expression level in H1299 lung cancer cells, indicating a role of CHIP on SIRT6 degradation, which is opposite to that observed in breast cancer cells [55]. Thus, it is speculated that CHIP may have cell-type specific effects on SIRT6 expression. In addition, other E3 ubiqutin ligases such as Skp2, ITCH, and Iduna were also suggested to participate SIRT6 ubiquitylation in lung cancer cells in this study.

cAMP signaling system was found to decrease SIRT6 expression by PKA-dependent inhibition of Raf-MEK-ERK pathways in this study. This finding is supported by the results that inhibition of PKA by either treatment with H89 or expression of dominant

negative PKA abolished the SIRT6 reduction following activation of cAMP signaling in lung cancer cells. PKA is a cAMP-dependent protein kinase, and a major effector molecule activated by cAMP, together with nucleotide-gated ion channels and exchange protein activated by cAMP (Epac). PKA phosphorylate numerous protein targets including enzymes and transcription factors to regulate various cellular responses such as metabolism and gene expression [60]. Our result suggests that PKA phosphorylate specific target proteins to promote ubiqutylation of SIRT6, and thus, we screened signaling molecules that mediate the promotion. We found Ras-MEK-Erk signaling mediates SIRT6-reducing effect of cAMP signaling, which was corroborated by the results that inhibition of Erk by treatment with chemical inhibitors or expression of dominant negative mutant Erks reduced SIRT6 expression, that activation of Erk by expressing constitutively active MEK abolished SIRT6 reducing effect of PGE2, and PGE2 inhibited Ras-MEK-Erk signaling in PKA-dependent manner. PGE2 was found to inhibit activation of c-Raf by increasing inhibitory phosphorylation of c-Raf at serine 259 residue and by decreasing activating phosphorylation at serine 338 [61], which resulted in inhibition of downstream MEK-ERK signaling to reduce SIRT6 expression.

Our study showed that cAMP signaling inhibits Erk pathway for a short time (< 2h), but reduced expression SIRT6 is observed, continuously. The mechanism of delayed effect of Erk inhibition on SIRT6 expression is not known. However, we speculate that transient inhibition of Erk may induce a weak but continuous promotion of SIRT6 degradation.

In addition to Erk-dependent reduction of SIRT6, PKA is suggested to activate CREB to reduce SIRT6 expression by another mechanism, regulating expression of CRE containing genes via CREB. It is supported by the result that expression of dominant negative CREB or CRE decoy oligonucleotides increases SIRT6 expression levels before and after PGE2 treatment.

Finally we analyzed the effect of reduced expression of SIRT6 by cAMP signaling on apoptosis, and we found that cAMP signaling augments radiation-induced apoptosis of lung cancer cells by reducing SIRT6 expression. This finding is substantiated by the results that cAMP signaling augmented radiation induced apoptosis of lung cancer cells, that cAMP signaling reduced SIRT6 expression, that exogenous expression of SIRT6 abolished apoptosis-augmenting effect of cAMP signaling, and that exogenous

expression of SIRT6 reduced radiation-induced apoptosis. . We have shown that cAMP signaling regulates apoptosis of various cancer cells including lung cancer cells through multiple mechanisms such as controlling expression of Bcl-2 family proteins [22, 31, 46, 62], X-linked inhibitor of apoptosis protein [47], and the activity of ATM [63]. In this paper, we present a novel mechanism of cAMP signaling to regulate apoptosis by regulating SIRT6 expression. Our finding agrees with the observed massive apoptosis in cancer cells following SIRT6 overexpression [64]. They describe that cell death requires the mono-ADP-ribosyltransferase of SIRT6 and is mediated by the activation of both the p53 and p73. However, H1299 lung cancer cells we used are lack of P53, so the molecular mechanism for SIRT6 to augment apoptosis of lung cancer cells needs to be further investigated.

SIRT6 has known to act tumor suppressor in hepatocellular carcinoma [65], or oncogene [37, 66], which is difference by cell type, but in our system, SIRT6 expression is increased in lung cancer cells, and SIRT6 maybe has oncogenic effect. There is a novel mechanism that decreases g-ray induced apoptosis by SIRT6 in lung cancer.

From this study, it is concluded that cAMP signaling systems reduces SIRT6 expression by promoting its ubiquitin-proteasome dependent degradation, that the degradation is mediated by PKA-dependent inhibition of ERK pathway, and that the reduced SIRT6 expression contributes to the augmented radiation-induced apoptosis by cAMP signaling in lung cancer cells. This study shows a novel mechanism for regulating SIRT6 expression and apoptosis by cAMP signaling, suggesting a potential mechanism for cAMP signaling to contribute to epigenetic control via histone decetylation in lung cancer cells.

Conclusion

This study aimed to examine how cAMP signaling regulate SIRT6 protein in non-small cell lung cancer cells, and the following conclusions were obtained.

1. cAMP signaling decreases SIRT6 protein expression by stimulating proteasome-dependent degradation of SIRT6 protein.

2. cAMP decreases SIRT6 expression via PKA/Raf/Mek/Erk dependent pathway.

3. cAMP signaling promotes radiation-induced apoptosis through SIRT6 depletion in lung cancer cells.

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국문 초록

cAMP 신호전달계는 물질 대사, 유전자 발현, 세포 성장, 그리고 세포 사멸 등 세포내의 여러 가지 기능을 조절하는 역할을 수행한다. 하지만, cAMP 신호전달계가 히스톤 수정 단백질을 포함하는 후성적 변화를 조절하는지 여부는 거의 연구가 이루어지지 않았다.

SIRT6 단백질은 sirtuin 단백질의 구성원으로서, NAD^+ 에 의존하는 히스톤 디아세틸 활성을 가지고 있다. SIRT6는 히스톤 H3의 라이신 9번, 56번 자리의 아세틸 기를 제거하고, 포도당 대사 항상성, 유전자 안정성, 그리고 세포 생장을 조절하지만, SIRT6 단백질의 발현을 조절하는 기전은 아직 명확히 밝혀지지 않았다. 그래서, 폐암세포주에서 cAMP 신호전달계가 히스톤 수정 단백질을 통제하는 메커니즘을 규명하기 위하여, 우리는 cAMP가 폐암세포주에서 SIRT6 단백질의 활성을 조절하여, 방사선 유도 세포사멸을 조절한다는 가정을 세웠다. 우리는 cAMP 신호전달계가 SIRT6 단백질 발현과 감마 방사선 우도 세포사멸에 대한 영향에 대해 연구를 수행하였다. 항시활성형 Gαs에 의한 cAMP 신호전달계의 활성화나, GPCR의 작용제인 prostaglandin E2 (PGE2), 그리고 isoproterenol, 또는 forskolin에 의해 비소세포폐암의 세포주인 H1299와 A549에서 SIRT6 단백질 발현이 SIRT6 mRNA 양의 변화없이 감소

하였다. cAMP는 SIRT6 단백질의 저하를 증대시켰고, 프로테오좀을 저하시키는 MG132에 의해 SIRT6 단백질 발현이 회복되었다. cAMP는 SIRT6 단백질의 유비퀴틴화를 증가시켰다. PKA를 저하시키는 H89의 처리, 또는 우성음성형 PKA의 발현에 의해서 cAMP에 의한 SIRT6 단백질의 감소가 회복되었다. PGE2 처리는 PKA에 의존적으로 c-Raf의 억제 인산화 잔기인 세린 259 부분을 활성화시켰고, 활성 인산화 잔기인 세린 388 부분을 감소시켰고, MEK-ERK 신호전달계의 활성을 저해시켰다. Erk 저해제의 처리나, 우성음성형 Erk의 발현은 SIRT6 단백질의 발현을 감소시켰고, 항시활성형 Erk를 발현시켰을 경우에는 PGE2에 의한 SIRT6 단백질의 감소가 회복되었다. cAMP 신호전달계는 폐암세포에서의 방사선 유도 세포사멸을 증가시키고, 이 반응은 SIRT6 단백질의 과발현을 통해서 감소되었다. 우리는 폐암세포주에서 cAMP 신호전달계가 SIRT6 단백질을 유비퀴틴/프로테오좀 의존 저해 과정을 통해서 감소시키고, 이는 PKA 의존하는 ERK 신호전달계의 저해 현상과 관련되어 있으며, SIRT6 단백질의 감소는 방사선 유도 세포사멸기전을 촉진한다고 결론지었다.

주요어 : SIRT6, 후성유전, cAMP 신호전달계, 히스톤 디아세틸화 단백질, PKA, c-Raf/Mek/Erk 신호전달, 세포사멸

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