Cyclic AMP Signaling Reduces Sirtuin 6 Expression in Non-small Cell Lung Cancer Cells by Promoting Ubiquitin-Proteasomal Degradation via Inhibition of the Raf-MEK-ERK (Raf/Mitogen-activated Extracellular Signal-regulated Kinase/Extracellular Signal-regulated Kinase) Pathway

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Background: cAMP signaling augments radiation-induced apoptosis of lung cancer cells. 
Results: cAMP signaling reduces the expression of sirtuin 6 deacetylase in non-small cell lung cancer cells by promoting ubiquitin-proteasomal degradation via inhibition of the Raf-MEK-ERK pathway.
Conclusion: cAMP signaling reduces sirtuin 6, which augments radiation-induced apoptosis in lung cancer cells.
Significance: cAMP signaling augments radiation-induced apoptosis by modulating epigenetic control.

The cAMP signaling system regulates various cellular functions, including metabolism, gene expression, and death. Sirtuin 6 (SIRT6) removes acetyl groups from histones and regulates genomic stability and cell viability. We hypothesized that cAMP modulates SIRT6 activity to regulate apoptosis. Therefore, we examined the effects of cAMP signaling on SIRT6 expression and radiation-induced apoptosis in lung cancer cells. cAMP signaling in H1299 and A549 human non-small cell lung cancer cells was activated via the expression of constitutively active Gos, plus treatment with prostaglandin E2 (PGE2), isoproterenol, or forskolin. The expression of sirtuins and signaling molecules were analyzed by Western blotting. Activation of cAMP signaling reduced SIRT6 protein expression in lung cancer cells. cAMP signaling increased the ubiquitination of SIRT6 protein and promoted its degradation. Treatment with MG132 and inhibiting PKA with H89 or with a dominant-negative PKA abolished the cAMP-mediated reduction in SIRT6 levels. Treatment with PGE2 inhibited c-Raf activation by increasing inhibitory phosphorylation at Ser-259 in a PKA-dependent manner, thereby inhibiting downstream MEK-ERK signaling. Inhibiting ERK with inhibitors or with dominant-negative ERKs reduced SIRT6 expression, whereas activation of ERK by constitutively active MEK abolished the SIRT6-depleting effects of PGE2. cAMP signaling also augmented radiation-induced apoptosis in lung cancer cells. This effect was abolished by exogenous expression of SIRT6. It is concluded that cAMP signaling reduces SIRT6 expression by promoting its ubiquitin-proteasome-dependent degradation, a process mediated by the PKA-dependent inhibition of the Raf-MEK-ERK pathway. Reduced SIRT6 expression mediates the augmentation of radiation-induced apoptosis by cAMP signaling in lung cancer cells.

Lung cancer is a malignant tumor arising from the respiratory epithelium. It is a leading cause of cancer death in both males and females worldwide and has the second highest incidence in women and developing countries (1). Lung cancer is divided into two morphologic groups: small cell lung cancer (SCLC)² and non-small cell lung cancer (NSCLC). NSCLC accounts for ~85% of all lung cancer cases (2). Lung cancer is treated by surgery, irradiation, and/or chemotherapy; however, the prognosis remains dismal, with a 5-year survival rate for NSCLC of around 15% (3). Thus, much research has been devoted to improving the efficiency of chemotherapy and radiotherapy based on a better understanding of the molecular mechanisms underlying cell proliferation and death; such research should bring substantial medical benefits, including longer survival and/or amelioration of symptoms (4).

² The abbreviations used are: SCLC, small cell lung cancer; NSCLC, non-small cell lung cancer; AC, adenylate cyclase; CREB, cAMP response element (CRE)-binding protein; Goα, stimulatory α subunit of G protein; Gα, the constitutively active mutant long form of the α subunit of stimulatory heterotrimeric GTP-binding protein; PKA, cAMP-dependent protein kinase; PPAR, poly(ADP-ribose) polymerase; qPCR, quantitative PCR; siRNA, small interfering RNA; SIRT6, sirtuin 6; HDAC, histone deacetylase; PGE2, prostaglandin E2; dn, dominant negative.
by divalent cations. Cyclic nucleotide phosphodiesterases degrade cAMP to 5’-AMP. cAMP binds to and activates cAMP-dependent protein kinase (PKA), exchange factor directly activated by cAMP (EPAC), and cyclic-nucleotide-gated ion channels (CNG) (5). Activated PKA phosphorylates enzymes to regulate both metabolism and transcription factors such as cyclic AMP response element binding protein (CREB). This in turn regulates the expression of other genes (6). cAMP signaling also modulates cancer cell death induced by anticancer drugs and γ-rays (7, 8).

Histone acetylation is one of the major mechanisms underlying the epigenetic regulation of gene expression and results in loosening of tightly packed heterochromatin to the more relaxed euchromatin. Histone acetylation status is determined by the balance between acetylation (by histone acetyltransferases) and deacetylation (by histone deacetylases (HDACs)) (9). The sirtuins, one of four families of HDAC, use nicotinamide adenine dinucleotide (NAD⁺) as a co-substrate. Mammals express seven sirtuin isoforms, each of which has a different substrate specificity and subcellular localization. The sirtuins are involved in a variety of cellular functions, including genomic stability, life span, aging, energy metabolism, and tumorigenesis (10). Sirtuin 6 (SIRT6) removes an acetyl group containing the epigenetic regulation of gene expression and results in loosening of tightly packed heterochromatin to the more relaxed euchromatin. Histone acetylation status is determined by the balance between acetylation (by histone acetyltransferases) and deacetylation (by histone deacetylases (HDACs)) (9).

Both cAMP signaling and SIRT6 are thought to be involved in regulating cancer cell death induced by anticancer drugs and ionizing radiation. Our own preliminary experiments suggested that cAMP signaling reduces the expression of SIRT6 in lung cancer cells. Thus, we hypothesized that cAMP modulates SIRT6 activity and regulates radiation-induced cell death in lung cancer cells. Here, we examined the effect and the underlying mechanism of cAMP signaling on SIRT6 protein expression and γ-radiation-induced apoptosis in lung cancer cells. We found that cAMP signaling induced the ubiquitin-dependent degradation of SIRT6, thereby reducing its levels within the cell. This process was mediated by the PKA-Raf-MEK-ERK pathways. We also showed that cAMP signaling augmented radiation-induced apoptosis in lung cancer cells, at least in part by down-regulating the expression of SIRT6 protein.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Reagents**—Human NSCLC cell lines H1299 and A549 (Korea Cell line Bank, Seoul, Korea), were cultured in Dulbecco’s modified Eagle’s medium (DMEM) for H1299 cells or RPMI 1640 for A549 cells containing 10% fetal bovine serum (Weligeon, Gyeongsan, Korea) and 100 units/ml penicillin/streptomycin. The cells were incubated in a 5% CO₂ incubator at 37 °C. Isoproterenol, SP600125, PD0325901, and DMSO were purchased from Sigma. PGE₂, forskolin, MG132, PD98059, and SB600125 were purchased from Cayman Chemical (Ann Arbor, MI). The FITC-annexin V apoptosis detection kit was purchased from BD Biosciences. Phenylmethanesulfonyl fluoride (PMSF), protease inhibitor mixture, sodium orthovanadate, and sodium fluoride were purchased from Roche Applied Science.

**Transient Transfection and Expression Plasmids**—H1299 and A549 cells were transfected with a pcDNA3.1 vector harboring a constitutively active mutant of long-form Goα containing a Glu-Glu tag (Goα-QL-EE) (Missouri S&T cDNA Resource Center, MO) using the calcium phosphate method. This mutant harbors a leucine residue instead of glutamate at 227 residues (the latter is essential for the intrinsic GTPase activity). A dominant-negative PKA (dnPKA) was a gift from Dr. G. Stanley McKnight (University of Washington) (15). Wild-type and dominant-negative CREBs (S133A, R287L) were gifts from Dr. Sahng-June Kwak (Dankook University, Cheonan, Korea). Constitutively active MEK1 was a gift from Dr. Pann-Ghill Suh (Ulsan National Institute of Science and Technology, Ulsan, Korea) (16). Wild-type and dominant-negative ERK1 and ERK2 (ERK1 K71R, and ERK2 K52R, respectively) were gifts from Drs. Melanie H. Cobb (University of Texas) (17). Wild-type and dominant-negative SIRT6 were gifts from Dr. Karin F. Chua (Stanford University) (18). The plasmids containing short hairpin RNA sequences targeting Goα, CHIP (the carboxyl terminus of Hsp70-interacting protein), ITCH, MDM2, Skp2, and scrambled shRNA (negative control) were purchased from Sigma. Iduna shRNA was a gift from Dr. Yun-II Lee (Samsung Advanced Institute of Technology, Seoul, Korea) (19). Decoy oligonucleotides for the CRE (CRE decoy) were prepared as described previously (20).

**Western Blot Analysis**—Western blotting was performed as previously described (21). Antibodies against SIRT1, -2, and -7, Skp2, ubiquitin, and p-CREB were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and antibodies against SIRT6, CHIP, Mdm2, p-c-Raf, c-Raf, p-MEK, MEK, p-ERK, ERK, CREB, p-p38, p38, p-JNK, JNK, poly(ADP-ribose) polymerases (PARP), caspase-3, and FLAG were purchased from Cell Signaling Technology (Beverly, MA). An antibody against the Glu-Glu tag was purchased from Covance (Princeton, NJ) and an antibody against β-actin was purchased from Sigma. Proteins were visualized using the Enhanced Chemiluminescence reagent (Menlo Park, CA), and blot images were acquired by an LAS-3000 luminescent image analyzer (Fuji, Tokyo, Japan). Band density was quantified using Multi Gauge v.2.3 software (Fuji) and expressed as a ratio relative to the control band density.

**Quantitative Reverse Transcription Polymerase Chain Reaction (qPCR)—**qPCR was performed in a C1000 thermal cycler (Bio-Rad) as previously described (22). The sequences of primers used for qPCR were as follows: SIRT6, 5’-CGAATTCGCA-CAACCACCTTT-3’ and 5’-CGGACGTACTGCTTTACACA-3’; CHIP, 5’-AGGCGAACAGCAGCAGTACAT-3’ and 5’-CTGTCCAGCCACAGGTATG-3’; IDUA, 5’-CTACAA-ACAGGAAGCAGAC-3’ and 5’-CTGAAGCTCCTTTTACACA-3’; ITCH, 5’-CCCTACGTTAGGTCACAGTAG-3’ and 5’-CTCCAAGCTGCAAAGTCAC-3’; and 5’-CTCCAAGCTGCAAAGTCAC-3’; MDM2, 5’-AACAGGAAAGCGAAC-3’ and 5’-CTGTCTCAGTGTTCCAAGTT-3’; GAPDH, 5’-ACCACAGTCCATG-CCATAC-3’ and 5’-
After 40 cycles of PCR, the average threshold cycle (Ct) values obtained from triplicate qPCR reactions were normalized against the Ct values for GAPDH.

Immunoprecipitation—H1299 cells transfected with GsQL or empty vector were lysed in lysis buffer containing 20 mM Tris-Cl (pH 7.5), 150 mM NaCl, 0.5% Nonidet P-40, 2 mM EDTA, 2 mM EGTA, 1 mM Na3VO4, 1 mM NaF, 1 mM PMSF, and a protease inhibitor mixture. The cell lysate (800 µg of protein) was precleared by incubation with protein G-agarose (Santa Cruz) for 1 h followed by centrifugation. The supernatant was then incubated with appropriate antibodies (1 µg) overnight at 4 °C on a rotator followed by incubation with 40 µl of protein G-agarose for 2 h. Bound proteins were then precipitated by centrifugation. The precipitate was washed three times before Western blot analysis with an anti-ubiquitin antibody.

Flow Cytometry—Lung cancer cells were exposed to γ-rays (10 gray) and then incubated for 24 h. The cells were then washed twice with phosphate-buffered saline and harvested by trypsinization and centrifugation at 3500 x g for 5 min at 4 °C. The cells were incubated in annexin V buffer containing FITC-annexin V and propidium iodide for 15 min. The fluorescence of 10,000 cells per sample was detected in a FACSCalibur flow cytometer (BD Biosciences).

Data Analysis—All experiments were repeated at least three times, and the data were expressed as the means ± S.E. Data were analyzed using a non-parametric Mann-Whitney U test. A p value < 0.05 was considered statistically significant.

RESULTS

cAMP Signaling Reduces Sirtuin 6 Expression

FIGURE 1. Gαs inhibits SIRT6 expression in lung cancer cells. A, effects of Gαs on the expression of sirtuin isoforms in H1299 human lung cancer cells. B, effects of Gαs knockdown on the expression of SIRT6. C, effects of cAMP signaling on the expression of SIRT6. D, effects of Gαs on the expression of SIRT6 in A549 human lung cancer cells. Lung cancer cells (H1299 and A549 cells) were transfected with EE-tagged GαsQL (GsQL) or vector pcDNA3.1 (V) using the calcium phosphate method and then incubated for 24 h. The cells were then harvested, homogenized, and analyzed by Western blotting. Gαs expression was knocked down by transfection of 10 µg of shRNA. Scrambled shRNA was used as a control (SC). The two arrowheads indicate long- and short-forms of Gαs proteins (8). H1299 cells were treated with 40 µM forskolin, 10 µM PGE2, 1 µM isoproterenol (ISO), distilled water (DW), or DMSO for 24 h before Western blotting analysis. An asterisk indicates a statistically significant difference compared with the respective control or vector-transfected cells (*, p < 0.05; Mann-Whitney U test).

TCCACCACCTGTGGCTGTA-3’. After 40 cycles of PCR, the average threshold cycle (Ct) values obtained from triplicate qPCR reactions were normalized against the Ct values for GAPDH.

Immunoprecipitation—H1299 cells transfected with GαsQL or empty vector were lysed in lysis buffer containing 20 mM Tris-Cl (pH 7.5), 150 mM NaCl, 0.5% Nonidet P-40, 2 mM EDTA, 2 mM EGTA, 1 mM Na3VO4, 1 mM NaF, 1 mM PMSF, and a protease inhibitor mixture. The cell lysate (800 µg of protein) was precleared by incubation with protein G-agarose (Santa Cruz) for 1 h followed by centrifugation. The supernatant was then incubated with appropriate antibodies (1 µg) overnight at 4 °C on a rotator followed by incubation with 40 µl of protein G-agarose for 2 h. Bound proteins were then precipitated by centrifugation. The precipitate was washed three times before Western blot analysis with an anti-ubiquitin antibody.

Flow Cytometry—Lung cancer cells were exposed to γ-rays (10 gray) and then incubated for 24 h. The cells were then washed twice with phosphate-buffered saline and harvested by trypsinization and centrifugation at 3500 x g for 5 min at 4 °C. The cells were incubated in annexin V buffer containing FITC-annexin V and propidium iodide for 15 min. The fluorescence of 10,000 cells per sample was detected in a FACSCalibur flow cytometer (BD Biosciences).

Data Analysis—All experiments were repeated at least three times, and the data were expressed as the means ± S.E. Data were analyzed using a non-parametric Mann-Whitney U test. A p value < 0.05 was considered statistically significant.
adenyl cyclase activator, forskolin, also reduced SIRT6 expression in cancer cells at 24 h after treatment (Fig. 1C). Expressing GαsQL also reduced SIRT6 protein in A549 lung cancer cells (Fig. 1D). Taken together, these results indicate that cAMP signaling reduces SIRT6 protein expression in lung cancer cells.

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cAMP Signaling Promotes Ubiquitin-Proteasome-dependent Degradation of SIRT6 in H1299 Cells—To investigate the mechanism by which cAMP signaling reduces SIRT6 expression, we next used quantitative RT-PCR to examine the effects of GαsQL on the expression of SIRT6 mRNA in H1299 cells. Expressing GαsQL did not significantly alter the levels of SIRT6 mRNA (Fig. 2A). Thus, we next examined the effect of GαsQL on SIRT6 degradation in H1299 cells. The results showed that expressing GαsQL in the presence of cycloheximide (an inhibitor of protein synthesis) promoted SIRT6 degradation (Fig. 2B). Because many proteins are ubiquitinated before proteasomal degradation, we next examined whether GαsQL affected SIRT6 ubiquitination. We found that the expression of GαsQL increased the amount of polyubiquitinated SIRT6 that was immunoprecipitated from H1299 cell lysates by an anti-SIRT6 antibody (Fig. 2D). Next, we examined whether MG132 affected the proteasomal degradation of
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SIRT6 after cAMP signaling activated by either PGE2 or forskolin. The results showed that MG132 completely abolished the PGE2- or forskolin-mediated reduction in SIRT6 expression (Fig. 3, A and B). To identify the ubiquitin E3 ligase involved in SIRT6 degradation, we next analyzed SIRT6 expression after knocking down the E3 ligases CHIP, iduna, ITCH, and Skp2 using specific shRNAs. E3 ligase knockdown resulted in basal and PGE2-induced levels of SIRT6 expression (Fig. 4C). Similarly, blocking the binding of CREB to its target genes by transfecting cells with a CRE decoy oligonucleotide increased SIRT6 expression, which was unaffected by PGE2 (Fig. 4D). The results indicate that cAMP signaling reduces SIRT6 expression in lung cancer cells by activating the PKA and CREB pathways.

**cAMP Signaling Reduces SIRT6 Expression in H1299 Cells by Inhibiting the ERK Pathway**—To study the signaling pathway that mediates the SIRT6-reducing effect of cAMP signaling, we first examined the time course of SIRT expression in PGE2-treated cells. Treating H1299 cells with PGE2 for 1 h led to a significant reduction in SIRT6 expression after 24 h, and treatment for 2 h reached a maximum reduction in SIRT6 expression (Fig. 5A). This suggests that activation of PGE2 signaling within 2 h after exposure is sufficient to induce a reduction of SIRT6 expression. To examine the signaling pathway that mediates the SIRT6-reducing effect of cAMP in more detail, we next analyzed the effect of mitogen-activated protein kinases (MAPKs) on SIRT6 expression after treatment with specific inhibitors. Treatment with the ERK inhibitor, PD98059, reduced SIRT6 expression in a manner similar to that of PGE2; however, treatment with a JNK inhibitor (SP600125) or a p38 inhibitor (SB203580) had no effect on SIRT6 expression (Fig. 5B). To confirm the effect of ERK signaling on SIRT6 expression, we next examined ERK signaling in H1299 cells by expressing dominant-negative ERKs. Expressing dominant-negative ERK1 (dnERK1) or ERK2 (dnERK2) reduced SIRT6 expression (Fig. 5C), whereas expressing constitutively active MEK (caMEK) increased SIRT6 expression and abolished the HDAC6-reducing effect of PGE2 in H1299 cells (Fig. 5D).

To study the signaling pathway involved in the SIRT6-reducing effects of cAMP, we next examined the role of PKA. PKA was inhibited by both a pharmacologic inhibitor (H89) and dnPKA, because H89 can inhibit other protein kinases as well as PKA. Inhibiting PKA with H89 or by expression of dnPKA increased the basal level of SIRT6 expression in H1299 cells and abolished the SIRT6-reducing effects of PGE2 (Fig. 4, A and B). Treatment with an EPAC-selective cAMP analogue (8-(4-chloro-phenylthio)-2′-O-methyladenosine-3′,5′-cyclic monophosphate) did not reduce SIRT6 expression, and knockdown of EPAC did not block SIRT6-reducing effect of PGE2 (data not shown). Because CREB is a well known downstream target of PKA, we next examined whether it plays a role in this process. CREB activation was inhibited by transfecting H1299 cells with dominant-negative CREBs (CREB S133A and CREB R287L), resulting in an increase in both basal and PGE2-induced levels of SIRT6 expression (Fig. 4C). Similarly, blocking the binding of CREB to its target genes by transfecting cells with a CRE decoy oligonucleotide increased SIRT6 expression, which was unaffected by PGE2 (Fig. 4D). The results indicate that cAMP signaling reduces SIRT6 expression in lung cancer cells by activating the PKA and CREB pathways.

**FIGURE 4. CAMP signaling inhibits SIRT6 expression in H1299 cells via the PKA and CREB pathways.** A, inhibiting PKA abolished the effects of Gα_oQL on SIRT6 expression. B, inhibiting PKA blocked the effects of PGE2 on SIRT6 expression. C, inhibiting CREB activation increased SIRT6 expression. D, inhibiting CREB binding to CRE increased SIRT6 expression. H1299 cells were transfected with Gα_oQL, a wild-type CREB (WT), dominant-negative CREBs (S133A, R287L), oligonucleotides (CRE decoy, CRE control; C), or respective control vectors (V) and then incubated for 24 h. The cells were then treated with 20 μM H89, 10 μM PGE2, or DMSO for 24 h before Western blot analysis. CREB phosphorylation (pCREB) was analyzed 30 min after exposure to PGE2. Asterisks (⁎) on the histograms indicate a statistically significant difference from the respective control cells (p < 0.05, Mann-Whitney U test).
to examine the effects of cAMP-mediated reductions in SIRT6 expression, we next examined γ-ray-induced apoptosis in lung cancer cells. Activation of cAMP signaling via expression of GaαQL increased the cleavage of caspase 3 and PARP, and the percentage of annexin V-positive cells in γ-ray-irradiated H1299 cells, which indicated an increase in apoptosis (Fig. 7, A and B). Co-expression of wild-type SIRT6 abolished these effects. SIRT6 expression alone reduced the amount of γ-ray-induced apoptosis, but expression of dominant-negative SIRT6 failed to reduce the apoptosis significantly (Fig. 7C). PGE2-mediated activation of cAMP signaling also increased the cleavage of caspase 3 and PARP, which was also blocked by expression of wild-type SIRT6 (Fig. 7D). Expressing Gaα in A549 cells also increased the γ-ray-induced cleavage of caspase 3 and PARP (Fig. 7E). These results suggest that cAMP signaling reduces SIRT6 expression in lung cancer cells, which in turn augments γ-ray-induced apoptosis.

DISCUSSION

Here we examined the effect of cAMP signaling on SIRT6 expression in lung cancer cells. We also examined the underlying molecular mechanisms and their functional significance. We found that 1) cAMP signaling reduced SIRT6 expression by promoting its degradation via the ubiquitin-proteasome pathway, 2) SIRT6 degradation is mediated by the PKA-dependent inhibition of the Raf-MEK-ERK pathways, and 3) reduced SIRT6 expression augments γ-ray-induced apoptosis of NSCLC cells (Fig. 8).

Our finding that cAMP signaling reduces SIRT6 expression in lung cancer cells was supported by experiments showing that SIRT6 expression was reduced after activation of cAMP signaling via expression of constitutively active Gaα (which stimulates ACs) by treatment with Gaα-coupled receptor agonists (PGE2...
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A

| Relative amount (ratio) | 3 | 2 | 1 | 0 |
|------------------------|---|---|---|---|
| SIRT6                  | V | V | V | + |
| GasQL                  |  + | + | + | + |
| γ-Ray                  |   |   |   |   |

B

| Annexin V (+) cells (ratio) | 4 | 3 | 2 | 1 |
|-----------------------------|---|---|---|---|
| SIRT6                       | V | V | V | + |
| GasQL                       |  + | + | + | + |
| γ-Ray                       |   |   |   |   |

C

| Relative amount (ratio) | 1.2 | 1.0 | 0.8 | 0.6 | 0.4 | 0.2 | 0.0 |
|-------------------------|-----|-----|-----|-----|-----|-----|-----|
| dnSIRT6                 | V   | V   | V   | +   | +   | +   | +   |
| SIRT6                   |  +  | +   | +   | +   | +   | +   | +   |
| γ-Ray                   |   + | +   | +   | +   | +   | +   | +   |

D

| Relative amount (ratio) | 3 | 2.5 | 2.0 | 1.5 | 1.0 | 0.5 | 0.0 |
|-------------------------|---|-----|-----|-----|-----|-----|-----|
| PGE2                    | V | V   | V   | +   | +   | +   | +   |
| SIRT6                   |  + | +   | +   | +   | +   | +   | +   |
| γ-Ray                   |   | +   | +   | +   | +   | +   | +   |

E

| PARP                     |  |  |  |  |  |  |  |
|--------------------------|---|---|---|---|---|---|---|
| Cleaved caspase-3       |  |  |  |  |  |  |  |
| SIRT6                   |  |  |  |  |  |  |  |
| GasQL                   |  |  |  |  |  |  |  |
| β-Actin                 |  |  |  |  |  |  |  |

Annexin V-FITC

γ-Ray

Propidium iodide

PARP

Cleaved caspase-3

p-CREB

SIRT6

SIRT6-Flag

β-Actin

GosQL

γ-Ray
and isoproterenol) or by treatment with an adenylyl cyclase activator, forskolin, in H1299 and A549 NSCLC. cAMP signaling also increased SIRT7 expression but did not affect the expression of other SIRT isoforms, suggesting that cAMP has an isofom-specific effect. Several molecules, such as microRNAs and peroxisome proliferator-activated receptor γ, were reported to regulate SIRT6 expression (23, 24), and cAMP signaling also stimulates HDAC4 activity (25). However, the effect of cAMP signaling on SIRT6 expression is unclear. Here we show for the first time that cAMP signaling regulates SIRT6 expression in lung cancer cells. Thus, cAMP signaling appears to regulate gene expression by modulating deacetylation via HDACs as well as by activating transcription factors such as CREB.

We also showed that cAMP signaling reduces SIRT6 expression by promoting the degradation of SIRT6 via the ubiquitin-proteosome pathway. Although cAMP signaling did not decrease SIRT6 mRNA levels, it increased the ubiquitination of SIRT6. By contrast, inhibiting the proteasome abolished the cAMP-mediated proteasomal degradation of SIRT6. Several studies show that SIRT6 expression is regulated by ubiquitin-proteasome-dependent degradation (26–28). cAMP regulates the ubiquitin-proteasome degradation of several proteins by controlling ubiquitin E3 ligases, including atrogin-1 (29), NEDD4L (30), and SCF-type ubiquitin E3 ligase (31). We also found that the ubiquitin E3 ligases MDM2, CHIP, iduna, ITCH, and NEDD4L (30), and SCF-type ubiquitin E3 ligase (31). We also showed that cAMP signaling reduces SIRT6 expression, 3) that exogenous expression of SIRT6 abolishes the cAMP-reducing effect of PGE2, and that PGE2 inhibited Raf-MEK-ERK signaling in a PKA-dependent manner. PGE2 inhibited c-Raf activation by increasing the inhibitory phosphorylation of c-Raf at Ser-259 and by reducing activating phosphorylation at Ser-338. This, in turn, inhibited downstream MEK-ERK signaling, thereby reducing SIRT6 expression.

These results agree with those published in previous reports showing that cAMP increases inhibitory Raf-1 phosphorylation at Ser-259 and reduces activating Raf-1 phosphorylation at Ser-338 in a PKA-dependent manner, thereby inducing ERK deactivation (33, 34). ERK activates SIRT1 in chondrocytes (35) and induces SIRT1 expression in human endothelial cells (36); however, the effect of ERK on SIRT6 remains unknown. The present study showed that inhibiting the Raf-MEK-ERK pathway also reduces SIRT6 expression in lung cancer cells. The Ras-ERK cascade inhibits the ubiquitination and degradation of GATA3 protein during T cell differentiation (37); thus it is plausible that ERK also inhibits the ubiquitination and degradation of SIRT6 in lung cancer cells. In addition to the ERK-dependent reduction of SIRT6 expression, we found that PKA activates CREB and reduces SIRT6 expression by regulating the expression of CRE-containing genes. The expression of dominant-negative CREB or CRE decoy oligonucleotides increased SIRT6 expression both before and after PGE2 treatment. Thus, cAMP signaling seems to reduce SIRT6 expression by promoting its proteasomal degradation and by regulating its gene expression via CREB.

Finally, we examined the effect of reduced SIRT6 expression on apoptosis. Reduced SIRT6 expression mediated augmentation of radiation-induced apoptosis in lung cancer cells by cAMP signaling. Experiments showed that 1) cAMP signaling augmented radiation-induced apoptosis in lung cancer cells, 2) that cAMP signaling reduced SIRT6 expression, 3) that exogenous expression of SIRT6 abolished the cAMP signaling-mediated effects on apoptosis, and 4) that exogenous expression of cAMP signaling reduced SIRT6 expression via PKA-dependent inhibition of the Raf-MEK-ERK pathways. We found that inhibiting PKA by either treatment with a PKA inhibitor or by expression of dominant-negative PKA abolished the cAMP-mediated reduction in SIRT6 expression in lung cancer cells and that EPAC was not involved in the reduction of SIRT6. This finding indicates that cAMP signaling reduced SIRT6 expression via PKA (32). The results suggest that PKA phosphorylates specific target proteins to promote the ubiquitination of SIRT6; thus we tried to identify the signaling molecules that mediate this effect. We found that the cAMP-mediated reduction in SIRT6 expression is mediated via the Raf-MEK-ERK signaling pathways. This was confirmed by our findings that inhibiting ERK reduced SIRT6 expression, that activating ERK abolished the SIRT6-reducing effect of PGE2, and that PGE2 inhibited Raf-MEK-ERK signaling in a PKA-dependent manner. PGE2 inhibited c-Raf activation by increasing the inhibitory phosphorylation of c-Raf at Ser-259 and by reducing activating phosphorylation at Ser-338. This, in turn, inhibited downstream MEK-ERK signaling, thereby reducing SIRT6 expression.
SIRT6 reduced the level of radiation-induced apoptosis. We previously showed that cAMP signaling regulates apoptosis in various cancer cells, including lung cancer cells, by modulating the expression of Bcl-2 family proteins (8), the expression of X-linked inhibitor of apoptosis protein (22), and the activity of ataxia-telangiectasia mutated (ATM) protein kinase (21). Here, we describe a novel mechanism through which cAMP signaling augments apoptosis, reducing SIRT6 expression. The anti-apoptotic effect of SIRT6 suggests that it may have potentially oncogenic roles in the tumorigenesis and progression of lung cancer. Our findings support those of a recent report showing that SIRT6 functions as an oncogene in the murine epidermis (38) prostate cancer (39).

Novel approaches to obtain antiproliferative effects or apoptosis-inducing effects of various cancer cells by controlling cAMP signaling are vigorously investigated (40), and many HDAC inhibitors are explored to develop anti-cancer drugs (41). This study might contribute to the development of new strategies to improve cancer radiotherapy by modulating the activity of cAMP signaling and HDACs.

In conclusion, the present study showed that 1) cAMP signaling reduces SIRT6 expression by promoting its degradation via the ubiquitin-proteasome pathway, 2) that this process is mediated by PKA-dependent inhibition of the ERK pathway, and 3) that reduced SIRT6 expression mediates the augmentation of radiation-induced apoptosis by cAMP signaling in lung cancer cells. Thus, we have identified a novel mechanism by which cAMP signaling regulates SIRT6 expression and apoptosis in lung cancer cells, suggesting a regulatory role for cAMP signaling in epigenetic control of gene expression.

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