Docosahexaenoic Acid Induces Cell Death through Downregulation of Hedgehog Signaling via Surt6 Activation in Human EGFR Mutant Non-Small Cell Lung Cancer

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Abstract

Omega-3 polyunsaturated fatty acids (ω3-PUFAs), including docosahexaenoic acid (DHA), have been shown to exert anticancer effects by inducing apoptotic cell death. However, the mechanism for DHA-induced cell death in lung cancer is not fully understood. Here, we show that DHA induces apoptosis in two human EGFR mutant non-small cell lung cancer (NSCLC) cell lines, and that DHA-induced cell death is accompanied by SIRT6 activation and attenuated Hedgehog (Hh) signaling. Knockdown of SIRT6 using siRNAs inhibited DHA-induced apoptosis, whereas SIRT6 overexpression increased apoptotic cell death. DHA-induced SIRT6 activation was associated with downregulation of Hh signaling, and knockdown of SIRT6 resulted in augmentation of Hh signaling. Pretreatment of NSCLC cells with a Smoothened agonist prevented DHA-induced decreases in the levels of Hh signaling proteins and increases in cleaved PARP levels. Moreover, endogenous production of ω3-PUFAs in PC9 cells via fat-1 expression resulted in elevated SIRT6 levels and reduced levels of Hh signaling molecules, including Gli, following DHA treatment. Overall, these results implicate that ω3-PUFAs induce apoptosis by downregulating Hh signaling via SIRT6 activation in human EGFR mutant NSCLC cells. These findings suggest that ω3-PUFAs potentially represent an effective therapy for the chemoprevention and treatment of NSCLC.

Keywords: docosahexaenoic acid; apoptosis; SIRT6; Hedgehog signaling; non-small cell lung cancer cells
1. Introduction

Sirtuin 6 (SIRT6) is one of seven members of the sirtuin family[1], which consists of mammalian homologs of the yeast (Saccharomyces cerevisiae) protein silent information regulator-2[2]. SIRT6 is localized in the nucleus[1] and has two types of enzymatic activities: mono-adenosine diphosphate ribosyltransferase[3] and deacetylase[4] activities, both of which are dependent on nicotinamide adenine dinucleotide[5]. SIRT6 regulates various cellular processes, including telomere maintenance, repair of damaged DNA, aging, immunity, and glucose homeostasis, and is important in cancer[3, 6-9]. SIRT6 deficiency in mice leads to developmental and metabolic abnormalities as well as a shorter lifespan, whereas SIRT6 activation is associated with a longer lifespan[8]. However, several studies have shown that SIRT6 activation can also cause cell death; for example, SIRT6 acts as a tumor suppressor in immortalized mouse embryonic fibroblasts by modulating aerobic glycolysis[10], and SIRT6 overexpression enhances apoptosis via activation of both p53 and p73 in multiple cancer cell lines[11].

SIRT6 regulates the Hedgehog (Hh) signaling pathway[5], which plays a crucial role in controlling stem cell maintenance[12], cell differentiation, cell proliferation[13], and tissue polarity[14] in vertebrate embryonic development. Alteration of Hh signaling promotes tumorigenesis in advanced basal cell carcinoma and medulloblastoma[15, 16]. Moreover, Hh signaling is activated in various cancers, including lung, breast, brain, and prostate cancer[17]. In the absence of Hh ligands (Sonic Hh, Indian Hh, or Desert Hh), activation of Hh signaling is prevented by inhibition of Smoothened (SMO), a seven-pass transmembrane protein, by the 12-pass transmembrane receptor Patched 1 (PTCH1)[18]. Upon binding of Hh ligands to PTCH1, SMO is translocated to the primary cilium, where it associates with the GLI family zinc finger 1 (GLI1) transcription complex, which is finally transported to the nucleus to regulate expression of target genes (encoding GLI1, PTCH1, cyclin D1, and Bcl-2)[18-20].

Docosahexaenoic acid (DHA), an omega-3 polyunsaturated fatty acid (ω3-PUFA), shows anticancer effects against several types of cancer by various mechanisms, including regulation of AKT/mechanistic target of rapamycin[21, 22], mitogen-activated protein kinase[23, 24], and signal transducer and activator of transcription-3 signaling[23]. Although DHA has been shown to induce apoptosis through various pathways [24-26], it remains unknown whether Hh signaling and SIRT6 are involved in DHA-induced cell death.

In the present study, we investigated the mechanisms of DHA-induced cell death in human epidermal growth factor receptor (EGFR) mutant non-small cell lung cancer (NSCLC) cells. We observed that DHA decreased cell
viability and induced apoptosis of NSCLC cells. Additionally, we demonstrated that DHA stimulated SIRT6 expression, which resulted in downregulation of Hh signaling, and eventually led to apoptosis. Our results show that DHA induces apoptotic cell death through Hh signaling suppression via regulation of SIRT6. These findings imply that ω3-PUFAs potentially represent an effective therapy for the chemoprevention and treatment of human EGFR mutant NSCLC.

2. Results

2.1. DHA reduces cell viability in human EGFR mutant NSCLC PC9 and H1975 cells

Previous studies have shown that DHA decreases the viability of various cancer cells[24-26]. We confirmed that DHA also reduces the viability of two NSCLC cell lines, PC9 and H1975. Cells were cultured with various concentrations of DHA for 24 h, and cell viability was measured using the MTT assay. DHA treatment significantly decreased the viability of PC9 and H1975 cells in a dose-dependent manner (Figure 1A).

To determine whether the reduced viability of PC9 and H1975 cells following DHA treatment resulted from apoptosis, cells were assessed for apoptotic changes. DHA induced dose-dependent increases in the expression levels of cleaved poly(ADP-ribose) polymerase (PARP) and caspase 3, which are widely used apoptotic markers (Figure 1B). DHA treatment also resulted in attenuation of Bcl-2 protein levels and stimulation of Bax protein levels (Figure 1B). In addition, TUNEL staining, which was used to detect apoptotic nuclear DNA breaks[27], showed that DHA treatment caused a significant increase in the number of TUNEL-positive cells (Figure 1C). Finally, fluorescence-activated cell sorting (FACS) analysis was performed to assess the effect of DHA treatment on the proportion of hypodiploid (sub-G1) cells. DHA treatment resulted in a dose-dependent increase in the proportion of cells in sub-G1 phase (Figure 1D). Together, these results suggest that DHA induces apoptosis in human NSCLC cells.

2.2. Activation of SIRT6 is responsible for DHA-induced apoptotic cell death in human EGFR mutant NSCLC PC9 and H1975 cells

Because SIRT6 is related to cell death[8, 9], we investigated whether the observed induction of apoptotic cell death by DHA was related to SIRT6. To this end, we first analyzed the protein expression levels of SIRT6 in DHA-treated PC9 and H1975 cells. DHA markedly increased SIRT6 protein levels in a dose- and time-dependent
manner, as assessed by immunoblotting (Figure 2A, B). Moreover, DHA increased SIRT6 levels in both the cytoplasm and the nucleus, as assessed by immunostaining (Figure 2C). These results imply that DHA activates SIRT6 in EGFR mutant NSCLC cells.

To examine the role of SIRT6 in DHA-induced apoptotic cell death, we knocked down SIRT6 using small interfering RNAs (siRNAs). DHA dramatically increased the levels of cleaved PARP (Fig. 3A, upper) and the number of detached cells (Fig. 3A, lower), which could be partially reversed by treatment with non-targeting control siRNA. To confirm these findings, we also tested the effect of SIRT6 overexpression on DHA-induced apoptotic cell death; consistent with our previous result, we found that transient transfection of PC9 and H1975 cells with a SIRT6 expression vector, but not a control vector, resulted in significant increases in cleaved PARP levels and the number of detached cells after DHA treatment (Figure 3B). Together, these observations clearly demonstrate that DHA-induced apoptosis is mediated by activation of SIRT6.

2.3. DHA-induced SIRT6 activation is associated with downregulation of Hh signaling

Because recent reports have suggested that SIRT6 regulates Hh signaling[5], and because DHA can also modulate Hh signaling[28], we examined the potential involvement of Hh signaling in SIRT6-mediated apoptosis induced by DHA. Firstly, to confirm that DHA modulates Hh signaling in NSCLC cells, the mRNA and protein expression levels of Hh signaling molecules were assessed by reverse transcription-PCR (RT-PCR) and western blot analysis. Treatment of PC9 and H1975 cells with DHA induced a decrease in the levels of mRNAs encoding Indian Hedgehog (IHH), PTCH1, SMO, and GLI1 (Figure 4A) and a dose-dependent decrease in the protein expression levels (Figure 4B) of these Hh signaling molecules, indicating that DHA causes downregulation of the Hh signaling pathway in EGFR mutant NSCLC cells.

Next, we sought to investigate the relationship between SIRT6 and Hh signaling modulated by DHA. Firstly, we assessed the effect of SIRT6 overexpression on Hh signaling by transfecting cells with a SIRT6 expression vector and analyzing the protein levels of IHH, PTCH1, SMO, and GLI1. Western blotting revealed that SIRT6 overexpression decreased IHH, PTCH1, SMO, and GLI1 protein levels (Figure 4C). In addition, inhibition of SIRT6 using siRNAs resulted in significant elevation of the levels of Hh signaling molecules (Figure 4D). Collectively, our findings demonstrate that elevated SIRT6 activation in DHA-treated PC9 and H1975 NSCLC cells leads to downregulation of Hh signaling.
2.4. Inhibition of Hh signaling by DHA treatment causes apoptosis

To test whether Hh signaling was related to apoptosis, PC9 and H1975 cells were incubated with DHA in the presence and absence of a SMO agonist, SAG, and the levels of apoptotic markers were examined by western blot analysis. Pretreatment of the cells with SAG increased the protein expression levels of IHH, PTCH1, SMO, and GLI1 (Fig. 4E), and reversed the decrease in the protein levels of Hh signaling molecules triggered by DHA (Figure 4F). Moreover, treatment of the cells with both SAG and DHA prevented DHA-induced apoptosis, as assessed by cleaved PARP levels (Figure 4F). These results imply that DHA induces apoptosis by inhibiting Hh signaling.

2.5. Fat-1 gene expression increases expression of SIRT6 and decreases expression of Hh signaling molecules

To unveil the effects of endogenous ω3-PUFAs on apoptosis via SIRT6-mediated Hh signaling, we investigated the effect of fat-1 expression on SIRT6 and Hh signaling molecule expression; fat-1 gene encodes an ω3-desaturase that catalyzes the conversion of ω6-PUFAs to ω3-PUFAs[29]. Stable transfection of PC9 cells with a fat-1 expression vector (f-PC9 cell) resulted in a significant increase in SIRT6 levels and a significant reduction in the levels of Hh signaling molecules, compared with cells transfected with a control vector (c-PC9 cell) (Figure 5). These observations reveal that endogenous ω3-PUFAs (expression of fat-1 gene) also causes induction of SIRT6 and reduces the levels of Hh signaling molecules, including Gli, in PC9 cells.
3. Discussion

Several studies have suggested that DHA exerts anticancer effects by inducing apoptotic cell death, and several signaling pathways have been reported to be involved in this process [30, 31]. In the present study, we report for the first time that DHA-induced apoptosis in human EGFR mutant NSCLC is associated with the ability of DHA to trigger SIRT6 activation, which results in downregulation of Hh signaling (Figure 6). Our results showed that DHA decreased the viability of PC9 and H1975 cells (Figure 1A), decreased expression of the anti-apoptotic protein Bcl-2 (Figure 1B), elevated expression of cleaved PARP and caspase 3 (Figure 1B), and increased the number of TUNEL-positive cells (Figure 1C). DHA also increased the populations of PC9 and H1975 cells with fractional DNA content (sub-G1). Importantly, the increase in the proportion of sub-G1-phase cells was mirrored by diminished proportions of cells in other cell cycle phases (Figure 1D), suggesting that apoptosis, rather than induction of cell cycle arrest, was primarily responsible for the DHA-induced decrease in cell growth. In agreement with this observation, DHA has been demonstrated to suppress growth of H1299 NSCLC cells and SiHa cervical cancer cells via induction of apoptotic cell death rather than cell cycle arrest[24].

One of the more significant findings of this study is that activation of SIRT6 plays a crucial role in the induction of apoptotic cell death by DHA. SIRT6 is considered an essential regulator for cell survival[32], however, several recent studies have shown that SIRT6 can also act a tumor suppressor [33], although the roles of SIRT6 activation in cancer remain controversial. In our system, DHA stimulated SIRT6 activation (Figure 2A, B), which resulted in apoptosis (Figure 3A, B). This observation is supported by previous reports, which showed that SIRT6 decreases transcriptional activity at forkhead box O3 (FOXO3a) and c-Jun promoters, which regulate genes such as AKT and GSK-3β, promoting apoptotic cell death [34, 35]. It is possible that the mechanism for DHA-induced apoptosis involves FOXO3a or c-Jun. Further investigation of the mechanism for SIRT6 activation-induced apoptosis resulting from DHA treatment is therefore required.

Our results revealed that DHA-induced apoptosis is regulated by SIRT6-mediated Hh signaling in PC9 and H1975 cells (Figure 3), providing novel insights into the mechanism by which SIRT6 activation triggered by DHA treatment exerts pro-apoptotic effects. DHA is known to affect Hh signaling; for example, it has been reported that DHA promotes exon development and cell survival in hippocampal neurons, neural stem cells, and human myeloma cells by promoting Hh signaling[28]. Endogenous production of ω3-PUFAs using fat-1-stable PC9 cells (f-PC9) increased SIRT6 expression and decreased Hh signaling molecules including Gli. irregulated in fat-1-
stable PC9 cells (f-PC9) (Figure 5). Therefore, our findings strongly suggest that ω3-PUFAs may modulate SIRT6 and Hh signaling molecules in EGFR mutant NSCLC cells. However, the mechanism by which SIRT6 activation downregulates Hh signaling to mediate DHA-induced cell death in human EGFR mutant NSCLC cells remains unclear. One potential molecule that may mediate this process is nuclear factor-kappa B (NF-κB). NF-κB plays a crucial role in driving the expression of genes that regulate apoptosis, cellular senescence, and inflammation [36]. SIRT6 has been shown to interact with the RELA subunit of NF-κB and promote deacetylation of NF-κB target gene promoters, leading to inhibition of NF-κB in brain tumor cells [37]. Thus, DHA-mediated increases in SIRT6 expression may result in inhibition of Hh signaling via interaction with NF-κB. We are currently undertaking further investigation of the mechanism for the downregulation of Hh signaling via DHA-induced SIRT6 activation in PC9 cells.

In summary, our study indicates for the first time that DHA induces apoptosis in human EGFR mutant NSCLC cells by increasing SIRT6 levels and subsequently decreasing the levels of Hh signaling molecules (IHH, PTCH1, SMO, and GLI1) (Figure 6). Our results provide important evidence to support the potential use of ω3-PUFAs as anticancer agents against human EGFR mutant NSCLC cells.
4. Materials and Methods

4.1. Cell culture

Human NSCLC PC9 and H1975 cells were kindly offered from professor Jeong-Eun Lee (Chungnam National University Hospital, Daejeon, Korea) and cultured in RPMI 1640 medium supplemented with 10% heat inactivated fetal bovine serum and antibiotics. The cells were maintained at 37°C in 5% CO₂, 95% air incubators.

4.2. Chemicals and antibodies

DHA was dissolved in absolute ethanol and added to the medium to final concentrations as described in each experiment. Cells grown to 70% confluence were switched to serum-free medium and the culture was allowed to expand for 24 h before giving any treatment. For SMO actiator 3-Chloro-N-[trans-4-(methylamino)cyclohexyl]-N-[3-(4-pyridinyl)phenyl]methyl]benzo[b]thiophene-2-carboxamide (SAG) treatment, the cultures were further incubated for 24 h at 37°C.

The following antibodies were used in this study. The antibodies against poly(ADP-ribose) polymerase (PARP) 1/2, Bax, Bcl-2 and β-actin were purchased from Santa Cruz; Indian hedgehog (IHH), PTCH1, SMO were from Abcam; Caspase-3 and GLI-1 were from Cell Signaling Technology; SIRT6 was from Sigma; goat anti-rabbit secondary antibodies were from Calbiochem; goat anti-rabbit conjugated with Tetramethylrhodamine (TRITC) were from Invitrogen.

4.3. Western blot analysis

The cells treated DHA were lysed in cold RIPA buffer (50 mM Tris-HCl at pH 7.4, 800 mM NaCl, 5 mM MgCl₂, 0.5% NP40) with protease inhibitor cocktail, and the supernatant was obtained. Cell lysates (30 μg) were separated by 6-15% sodium dodecyl sulfate-PAGE and electroblotted onto a polyvinylidene difluoride membrane. The blots were blocked in 5% nonfat dry milk (M/V) or bovine serum albumin (BSA) in tris-buffered containing 0.1% Tween 20 for 1 h, and incubated with 1:1000-1:5000 dilution of primary antibody for overnight at 4°C, followed by probed with goat anti-rabbit peroxidase-conjugated secondary antibodies. Enhanced chemiluminescence reagents were used to detect relevant proteins.
4.4. Assays for cell viability and apoptosis

Cell viability was examined by the Thiazoly Blue Tetrazolium Bromide (MTT). $6 \times 10^3$ cells were seeded onto 96-well plates and then treated with increasing concentrations for 24 h. At the end of treatment, MTT was added and cell viability was assessed by spectrophotometry.

Apoptosis was detected by the terminal deoxyribonucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay for measuring DNA nicking. DHA treated PC9 and H1975 cells were stained with the DeadEnd™ Fluorometric TUNEL System, and then visualized under a fluorescence microscope (Olympus iX70). For Sub-G1 proportion analysis, both attached cells and supernatant collected, stained with propidium iodide, and performed with flow cytometry (FACS) analysis with a FACSCalibur flow cytometer (BD Biosciences).

4.5. Immunocytochemistry

After being grown on coverslips in growth media, the cells were treated with DHA for 8 h. The cells were fixed with 4% paraformaldehyde, and then permeabilized with 1% Triton X-100 in phosphate buffered saline. After washing, fixed cells were blocked with 1% BSA for 1 h, and then incubated with SIRT6 antibodies, and TRITC-conjugated anti-rabbit secondary antibodies, and 4', 6-diamidino-2-phenylindole (DAPI) were used for 1 h. Images were visualized using an Olympus iX70 fluorescence microscope.

4.6. Transfection and transduction

Transient transfection was performed with control vector (pflag) or SIRT6 expression vector (a kindly gift from professor Byung-Hyun Park, Chonbuk National University Medical School, Jeonju, Korea), using Lipofectamine 3000 reagent as recommended by the vendor (Invitrogen).

For knockdown experiments using small interfering RNAs (siRNAs), siRNAs for SIRT6 (siSIRT6) or negative control siRNA (siNC) were transfected into cells using Lipofectamin RNAiMAX according to the manufacturer’s instructions (Invitrogen). After transfection, the cells were exposed to DHA for another 24 h. siRNAs against the following genes were used: non-targeting control siRNA, 5'-ACG UGA CAC GUU CGG AGA AUU-3'; SIRT6, 5'-AGU UCG ACA CCA CCU UUG A-3'.

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4.7. RNA isolation and reverse transcriptional polymerase chain reaction (RT-PCR)

Total RNA was isolated using Trizol reagent (Invitrogen) and 1 μg total RNA was reverse-transcribed into cDNA using M-MLV reverse transcriptase (ElpisBio) containing anchored oligo (dT). For RT-PCR, 1 μg cDNA was performed amplification for each 20 μL of HiPi PCR PreMix reaction system (ElpisBio). The RT reaction was incubated for 5 min at 94°C followed by denaturation followed by 35 cycles of segments of 94°C for 30 sec, primer annealing at 53-65°C for 30 sec and extension at 72°C for 30 sec. PCR products were analyzed by a 2% agarose gel with electrophoresis and photographed under UV transillumination. The primer used to amplify the sirt6, ihh, pch1, SMO, GLI-1 and gapdh are 5′-CCC GGA TCA ACG GCT CT A TC-3′ (forward) and 5′-GCC TTC ACC CTT TTG GGG G-3′ (reverse); 5′-TCC AGA AAC TCC GAG CGA TTT AAG-3′ (forward) and 5′-ACT TCC TGG CCA CTG GTT CA-3′ (reverse); 5′-CCACAGAAGCGCTCAAACA-3′ (forward) and 5′-CTG TAA TTT CGC CCC TTC C-3′ (reverse); 5′-ACG AGG ACG TGG AGG GCT G-3′ (forward) and 5′-CGC AGC GTA TCG GTA GTT CT-3′ (reverse); 5′-GAG ATG ATC CCA CAT CCT CAG TC-3′ (forward) and 5′-GCC ATC CAC AGT CTT CTG CTC-3′ (reverse).

4.8. Stable cell lines

PC9 cells were transfected with the fat-1 gene (pST180) or the control vector (pST128) using Lipofectamin 2000 reagent. After 4 h, the medium changed to media containing 10% FBS. Next day, 2,000 cells were replated onto 150 mm dishes and then selected with G418 (Geneticin) until generation of colonies.

4.9. Statistical analysis

Differences between groups were analyzed using the unpaired Student’s t-test. In all analyses, a P value less than 0.05 was considered statistically significant. *, ** and *** means P < 0.05, P < 0.01, and P < 0.001, respectively.

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**Author Contribution:** Soyeon Jeong and Kyu Lim designed execution of the experiments, interpretation of data, statistical analysis, and wrote the manuscript; Kaipeng Jing participated in the interpretation and analysis of data, and manuscript writing; Soyeon Shin participated in the f-PC9 stable cell experiments and interpretation of data; Soyeon Kim and Yoon-Seon Yoo participated in design of figure and the f-PC9 stable cell experiments; Young-Joo Jeon, Jun-Young Heo, Gi-Ryang Kweon, Seung-Kiel Park, and Jong-Il Park contributed interpretation of data and statistical analysis. All authors endorsed the submission of the final manuscript.

**Conflicts of Interest:** The authors declare no conflict of interest.
References

1. Michishita, E.; Park, J. Y.; Burneskis, J. M.; Barrett, J. C.; Horikawa, I., Evolutionarily conserved and nonconserved cellular localizations and functions of human SIRT proteins. *Mol Biol Cell* 2005, 16, (10), 4623-35.

2. Hanahan, D.; Weinberg, R. A., Hallmarks of cancer: the next generation. *Cell* 2011, 144, (5), 646-74.

3. Mao, Z.; Hine, C.; Tian, X.; Van Meter, M.; Au, M.; Vaidya, A.; Seluanov, A.; Gorbunova, V., SIRT6 promotes DNA repair under stress by activating PARP1. *Science* 2011, 332, (6036), 1443-6.

4. Michishita, E.; McCord, R. A.; Berber, E.; Kioi, M.; Padilla-Nash, H.; Damian, M.; Cheung, P.; Kusumoto, R.; Kawahara, T. L.; Barrett, J. C.; Chang, H. Y.; Bohr, V. A.; Ried, T.; Gozani, O.; Chua, K. F., SIRT6 is a histone H3 lysine 9 deacetylase that modulates telomeric chromatin. *Nature* 2008, 452, (7186), 492-6.

5. Piao, J.; Tsuji, K.; Ochi, H.; Iwata, M.; Koga, D.; Okawa, A.; Morita, S.; Takeda, S.; Asou, Y., Sirt6 regulates postnatal growth plate differentiation and proliferation via Ihh signaling. *Sci Rep* 2013, 3, 3022.

6. Preyat, N.; Leo, O., Sirtuin deacylases: a molecular link between metabolism and immunity. *J Leukoc Biol* 2013, 93, (5), 669-80.

7. Lin, Z.; Yang, H.; Tan, C.; Li, J.; Liu, Z.; Quan, Q.; Kong, S.; Ye, J.; Gao, B.; Fang, D., USP10 antagonizes c-Myc transcriptional activation through SIRT6 stabilization to suppress tumor formation. *Cell Rep* 2013, 5, (6), 1639-49.

8. Mostoslavsky, R.; Chua, K. F.; Lombard, D. B.; Pang, W. W.; Fischer, M. R.; Gellon, L.; Liu, P.; Mostoslavsky, G.; Franco, S.; Murphy, M. M.; Mills, K. D.; Patel, P.; Hsu, J. T.; Hong, A. L.; Ford, E.; Cheng, H. L.; Kennedy, C.; Nunez, N.; Bronson, R.; Frendewey, D.; Auerbach, W.; Valenzuela, D.; Karow, M.; Hottiger, M. O.; Hursting, S.; Barrett, J. C.; Guarente, L.; Mulligan, R.; Demple, B.; Yancopoulos, G. D.; Alt, F. W., Genomic instability and aging-like phenotype in the absence of mammalian SIRT6. *Cell* 2006, 124, (2), 315-29.

9. Zhong, L.; D'Urso, A.; Toiber, D.; Sebastian, C.; Henry, R. E.; Vadysirisack, D. D.; Guimaraes, A.; Marinelli, B.; Wikstrom, J. D.; Nir, T.; Clish, C. B.; Vaitheesvaran, B.; Iliopoulos, O.; Kurland, I.; Dor, Y.; Weissleder, R.; Shirihai, O. S.; Ellisen, L. W.; Espinosa, J. M.; Mostoslavsky, R., The histone deacetylase Sirt6 regulates glucose homeostasis via Hif1alpha. *Cell* 2010, 140, (2), 280-93.
10. Sebastian, C.; Zwaans, B. M.; Silberman, D. M.; Gymrek, M.; Goren, A.; Zhong, L.; Ram, O.; Truelove, J.; Guimaraes, A. R.; Toiber, D.; Cosentino, C.; Greenson, J. K.; MacDonald, A. I.; McGlynn, L.; Maxwell, F.; Edwards, J.; Giacosa, S.; Guccione, E.; Weissleder, R.; Bernstein, B. E.; Regev, A.; Shiels, P. G.; Lombard, D. B.; Mostoslavsky, R., The histone deacetylase SIRT6 is a tumor suppressor that controls cancer metabolism. Cell 2012, 151, (6), 1185-99.

11. Van Meter, M.; Mao, Z.; Gorbunova, V.; Seluanov, A., SIRT6 overexpression induces massive apoptosis in cancer cells but not in normal cells. Cell Cycle 2011, 10, (18), 3153-8.

12. Zhao, C.; Chen, A.; Jamieson, C. H.; Fereshteh, M.; Abrahamsson, A.; Blum, J.; Kwon, H. Y.; Kim, J.; Chute, J. P.; Rizzieri, D.; Munchhof, M.; VanArsdale, T.; Beachy, P. A.; Reya, T., Hedgehog signalling is essential for maintenance of cancer stem cells in myeloid leukaemia. Nature 2009, 458, (7239), 776-9.

13. Yu, J.; Carroll, T. J.; McMahon, A. P., Sonic hedgehog regulates proliferation and differentiation of mesenchymal cells in the mouse metanephric kidney. Development 2002, 129, (22), 5301-12.

14. Krauss, S.; Concordet, J. P.; Ingham, P. W., A functionally conserved homolog of the Drosophila segment polarity gene hh is expressed in tissues with polarizing activity in zebrafish embryos. Cell 1993, 75, (7), 1431-44.

15. Kimonis, V. E.; Goldstein, A. M.; Pastakia, B.; Yang, M. L.; Kase, R.; DiGiovanna, J. J.; Bale, A. E.; Bale, S. J., Clinical manifestations in 105 persons with nevoid basal cell carcinoma syndrome. Am J Med Genet 1997, 69, (3), 299-308.

16. Gorlin, R. J.; Goltz, R. W., Multiple nevoid basal-cell epithelioma, jaw cysts and bifid rib. A syndrome. N Engl J Med 1960, 262, 908-12.

17. Gupta, S.; Takebe, N.; Lorusso, P., Targeting the Hedgehog pathway in cancer. Ther Adv Med Oncol 2010, 2, (4), 237-50.

18. McMillan, R.; Matsui, W., Molecular pathways: the hedgehog signaling pathway in cancer. Clin Cancer Res 2012, 18, (18), 4883-8.

19. Rohatgi, R.; Milenkovic, L.; Corcoran, R. B.; Scott, M. P., Hedgehog signal transduction by Smoothened: pharmacologic evidence for a 2-step activation process. Proc Natl Acad Sci U S A 2009, 106, (9), 3196-201.

20. Hui, C. C.; Angers, S., Gli proteins in development and disease. Annu Rev Cell Dev Biol 2011, 27, 513-37.
21. Shin, S.; Jing, K.; Jeong, S.; Kim, N.; Song, K. S.; Heo, J. Y.; Park, J. H.; Seo, K. S.; Han, J.; Park, J. I.; Kweon, G. R.; Park, S. K.; Wu, T.; Hwang, B. D.; Lim, K., The omega-3 polyunsaturated fatty acid DHA induces simultaneous apoptosis and autophagy via mitochondrial ROS-mediated Akt-mTOR signaling in prostate cancer cells expressing mutant p53. *Biomed Res Int* 2013, 2013, 568671.

22. Kim, N.; Jeong, S.; Jing, K.; Shin, S.; Kim, S.; Heo, J. Y.; Kweon, G. R.; Park, S. K.; Wu, T.; Park, J. I.; Lim, K., Docosahexaenoic Acid Induces Cell Death in Human Non-Small Cell Lung Cancer Cells by Repressing mTOR via AMPK Activation and PI3K/Akt Inhibition. *Biomed Res Int* 2015, 2015, 239764.

23. Schley, P. D.; Brindley, D. N.; Field, C. J., (n-3) PUFA alter raft lipid composition and decrease epidermal growth factor receptor levels in lipid rafts of human breast cancer cells. *J Nutr* 2007, 137, (3), 548-53.

24. Jeong, S.; Jing, K.; Kim, N.; Shin, S.; Kim, S.; Song, K. S.; Heo, J. Y.; Park, J. H.; Seo, K. S.; Han, J.; Wu, T.; Kweon, G. R.; Park, S. K.; Park, J. I.; Lim, K., Docosahexaenoic acid-induced apoptosis is mediated by activation of mitogen-activated protein kinases in human cancer cells. *BMC Cancer* 2014, 14, 481.

25. Lim, K.; Han, C.; Dai, Y.; Shen, M.; Wu, T., Omega-3 polyunsaturated fatty acids inhibit hepatocellular carcinoma cell growth through blocking beta-catenin and cyclooxygenase-2. *Mol Cancer Ther* 2009, 8, (11), 3046-55.

26. Song, K. S.; Jing, K.; Kim, J. S.; Yun, E. J.; Shin, S.; Seo, K. S.; Park, J. H.; Heo, J. Y.; Kang, J. X.; Suh, K. S.; Wu, T.; Park, J. I.; Kweon, G. R.; Yoon, W. H.; Hwang, B. D.; Lim, K., Omega-3-polyunsaturated fatty acids suppress pancreatic cancer cell growth in vitro and in vivo via downregulation of Wnt/Beta-catenin signaling. *Panreatology* 2011, 11, (6), 574-84.

27. Bhat, N. R.; Zhang, P., Hydrogen peroxide activation of multiple mitogen-activated protein kinases in an oligodendrocyte cell line: role of extracellular signal-regulated kinase in hydrogen peroxide-induced cell death. *J Neurochem* 1999, 72, (1), 112-9.

28. Abdi, J.; Garssen, J.; Faber, J.; Redegeld, F. A., Omega-3 fatty acids, EPA and DHA induce apoptosis and enhance drug sensitivity in multiple myeloma cells but not in normal peripheral mononuclear cells. *J Nutr Biochem* 2014, 25, (12), 1254-62.

29. Lu, Y.; Nie, D.; Witt, W. T.; Chen, Q.; Shen, M.; Xie, H.; Lai, L.; Dai, Y.; Zhang, J., Expression of the fat-1 gene diminishes prostate cancer growth in vivo through enhancing apoptosis and inhibiting GSK-3 beta phosphorylation. *Mol Cancer Ther* 2008, 7, (10), 3203-11.
30. Hu, Y.; Sun, H.; Owens, R. T.; Gu, Z.; Wu, J.; Chen, Y. Q.; O'Flaherty, J. T.; Edwards, I. J., Syndecan-1-dependent suppression of PDK1/Akt/bad signaling by docosahexaenoic acid induces apoptosis in prostate cancer. *Neoplasia* 2010, 12, (10), 826-36.

31. Calviello, G.; Resci, F.; Serini, S.; Piccioni, E.; Toesca, A.; Boninsegna, A.; Monego, G.; Ranelletti, F. O.; Palozza, P., Docosahexaenoic acid induces proteasome-dependent degradation of beta-catenin, down-regulation of survivin and apoptosis in human colorectal cancer cells not expressing COX-2. *Carcinogenesis* 2007, 28, (6), 1202-9.

32. Lombard, D. B.; Schwer, B.; Alt, F. W.; Mostoslavsky, R., SIRT6 in DNA repair, metabolism and ageing. *Journal of internal medicine* 2008, 263, (2), 128-41.

33. Fukuda, T.; Wada-Hiraike, O.; Oda, K.; Tanikawa, M.; Makii, C.; Inaba, K.; Miyasaka, A.; Miyamoto, Y.; Yano, T.; Maeda, D.; Sasaki, T.; Kawana, K.; Fujayama, M.; Osuga, Y.; Fujii, T., Putative tumor suppression function of SIRT6 in endometrial cancer. *FEBS Lett* 2015, 589, (17), 2274-81.

34. Qi, W.; Fitchev, P. S.; Cornwell, M. L.; Greenberg, J.; Cabe, M.; Weber, C. R.; Roy, H. K.; Crawford, S. E.; Savkovic, S. D., FOXO3 growth inhibition of colonic cells is dependent on intraepithelial lipid droplet density. *J Biol Chem* 2013, 288, (23), 16274-81.

35. Sundaresan, N. R.; Vasudevan, P.; Zhong, L.; Kim, G.; Samant, S.; Parekh, V.; Pillai, V. B.; Ravindra, P. V.; Gupta, M.; Jeevanandam, V.; Cunningham, J. M.; Deng, C. X.; Lombard, D. B.; Mostoslavsky, R.; Gupta, M. P., The sirtuin SIRT6 blocks IGF-Akt signaling and development of cardiac hypertrophy by targeting c-Jun. *Nature medicine* 2012, 18, (11), 1643-50.

36. Tilstra, J. S.; Clauson, C. L.; Niedernhofer, L. J.; Robbins, P. D., NF-kappaB in Aging and Disease. *Aging Dis* 2011, 2, (6), 449-65.

37. Kawahara, T. L.; Michishita, E.; Adler, A. S.; Damian, M.; Berber, E.; Lin, M.; McCord, R. A.; Ongaigui, K. C.; Boxer, L. D.; Chang, H. Y.; Chua, K. F., SIRT6 links histone H3 lysine 9 deacetylation to NF-kappaB-dependent gene expression and organismal life span. *Cell* 2009, 136, (1), 62-74.
5. Figure legends

**Figure 1.** DHA induces apoptotic cell death in EGFR mutant NSCLC cells. (A) PC9 (left) and H1975 (right) cells were plated in 96-well plate at 6,000 cells per well. Then, cells were exposed to indicate doses of DHA for 24 h. Cell viability was measured by MTT assay. (B) The cells were incubated with DHA for 24 h, and the protein expression levels of PARP, Bax, Bcl-2, and Caspase-3 were determined by western blotting. (C) The cells were seeded in 12-well plate on coverslips, and exposed to DHA for 8 h, then subjected to the TUNEL assay. Cells were observed under an Olympus ix70 inverted fluorescence microscope (Scale bar, 50 μm). (D) PC9 and H1975 cells were treated with various dose of DHA for 24 h, and the proportion of Sub-G1 was examined by FACS analysis. The data shown are representative of 3 independent experiments with similar results.
Figure 2. DHA leads to elevation of SIRT6 expression. (A) The cells were incubated with increasing concentration of DHA for 24 h, and the expression levels of SIRT6 were assessed by immunoblotting. (B) PC9 and H1975 cells were treated with 60 μM DHA (PC9 cells) or 20 μM DHA (H1975 cells) for indicated time periods, the protein levels were monitored by western blot. Equal protein loading was confirmed by probing with an antibody against β-actin. (C) The cells were exposed to DHA for 8 h, and fixed using 4% paraformaldehyde. Then, the cells were incubated with a SIRT6 antibody, followed by DAPI nuclear counterstaining (Scale bar, 50 μm). Representative blot from more than 3 independent experiments are shown.
Figure 3. DHA-mediated apoptotic cell death is related to SIRT6 activation. (A) PC9 (left) and H1975 (right) cells were treated with siNC or siSIRT6. At 18 h after transfection, cells were incubated with the indicated doses of DHA for 24 h. Upper, cells were harvested and western analysis was performed. Lower, representative images of PC9 and H1975 cells treated with DHA (Scale bar, 200 μm). (B) PC9 (left) and H1975 (right) cells were transfected with pflag and SIRT6 for 18 h, then, the cells were treated with indicated doses of DHA for 24 h. Upper, the expression of SIRT6, PARP and β-actin was identified by western blotting. Lower, bright field microscopic images of PC9 and H1975 cells after incubation with the 60 μM DHA (PC9 cells) or 20 μM DHA (H1975 cells). Results are shown one representative images of three independent experiments.
Figure 4. DHA-induced apoptotic cell death is related to downregulation of Hh signaling via SIRT6 activation. (A) DHA-induced downregulation of Hh signal molecules mRNA expression including IHH, PTCH1, SMO, and GLI-1 were analyzed by RT-PCR analysis. (B) The cells were exposed to increasing concentrations of DHA for 24 h. Hh-related molecules (IHH, PTCH1, SMO, and GLI-1) were assessed by western blotting. (C) After SIRT6 expression vector was transfected in PC9 and H1975 cells transfected, Hh signal molecules were detected by western blot assay. (D) PC9 and H1975 cells were transfected with siNC or siSIRT6 and the protein expression levels of IHH, PTCH1, SMO, and GLI-1 were examined by immunoblotting. (E) Effect of SAG, SMO agonist, on Hh signal molecules expression. (F) PC9 and H1975 cells were treated with DHA in the presence and absence of SAG, and the apoptosis were examined by western blot analysis. All data shown are a representative of three independent experiments with similar results.
Figure 5. Endogenous ω3-PUFAs also augmented SIRT6 level and reduced Hh signaling molecules. Cell lysates were obtained from f-PC9, c-PC9 and a parental cells, respectively. SIRT6 and Hh signaling molecules including Gli were examined by western blot. β-Actin was served as an equal loading control.

Figure 6. Schematic model of DHA-induced apoptosis in human EGFR mutant NSCLC cells. The DHA-induced apoptosis was associated with its ability to SIRT6 activation and subsequent Hh signaling downregulation in human EGFR mutant NSCLC cells.