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Role of ATF3 in synergistic cancer cell killing by a combination of HDAC inhibitors and agonistic anti-DR5 antibody through ER stress in human colon cancer cells

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ABSTRACT

Histone deacetylase inhibitors (HDACIs) are promising agents for cancer therapy. However, the mechanism(s) responsible for the efficacy of HDACIs have not yet to be fully elucidated. Death receptor 5 (DR5) is a transmembrane receptor containing death domain that triggers cell death upon binding to TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) or agonistic anti-DR5 monoclonal antibody, and the combination of TRAIL/agonistic anti-DR5 monoclonal antibody and agents that increase the expression of DR5 is expected as a novel anticancer therapeutic strategy. Here we report that six different HDACIs activated endoplasmic reticulum (ER) stress sensor PERK and eIF2α and induced the ATF4/ATF3/CHOP pathway in p53-deficient human colon cancer cells. This resulted in an increased expression of DR5 on the cell surface and sensitized cells to apoptosis by agonistic anti-DR5 monoclonal antibody. Stress response gene ATF3 was required for efficient DR5 induction by HDACIs, and DR5 reporter assay showed that ATF3 play crucial role for the HDACIs-induced activation of DR5 gene transcription. These provide important mechanistic insight into how HDACIs exhibit pro-apoptotic activity in clinical anti-cancer treatments when they are used in combination with other therapeutic strategies.

1. Introduction

Histone deacetylase inhibitors (HDACIs) are a class of targeted therapeutics with promising anti-tumor efficacy that reverse aberrant epigenetic changes associated with cancer. HDACIs induce hyper-acetylation of DNA-bound histones or sequence-specific transcription factors, which can either increase or decrease the transcription of cancer-related genes [1–3]. It has also been shown that the treatment with HDACIs results in hyper-acetylation of chaperones such as HSP90, HSP70 and HSP40, which impairs the chaperone activity and leads to the accumulation of unfolded or misfolded proteins in endoplasmic reticulum (ER) [3–5]. This causes the activation of a specific cellular process called the unfolded protein response (UPR), which is mediated through at least three major pathways initiated by IRE1, PERK or ATF6 respectively, coordinating a temporal shut-down of protein translation and a complex gene transcriptional program to safeguard cell survival. Upon excessive or prolonged UPR, however, apoptosis is activated to eliminate faulty cells [6,7].

To date, several structurally distinct HDACIs have been developed and are classified into six groups, including short-chain fatty acids, hydroxamic acids, cyclic peptides, benzamides, electrophilic ketones, and hybrid molecules [8]. At present, an increasing number of HDACIs have been reported as candidate for stand-alone therapeutics or combination therapy with other agents. For instance, suberoylanilide hydroxamic acid (SAHA, also called Vorinostat) is a broad set and non-selective inhibitor of HDACs. After its approval for the treatment of cutaneous T cell lymphoma [9], SAHA is trying to find its value for solid tumors including thyroid cancer [10], breast cancer [11] and mesothelioma [12]. In colon cancer, it is reported that SAHA can induce sub-G1 arrest and apoptosis [13,14].

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Death receptor 5 (DR5) belongs to the TNF receptor family. Upon binding of its ligand TRAIL or agonistic antibody, DR5 mediates TRAIL-induced apoptosis through the formation of a death-inducing signaling complex [15]. DR5 is expressed more strongly in cancer cells than in normal cells [16], and mediates apoptosis selectively in cancer cells both *in vitro* and *in vivo*, with little or no effect on normal cells. However, clinical trials have shown that small therapeutic effects are observed when TRAIL or TRAIL agonistic monoclonal antibodies specific for DR5, such as DJR2-2 [17,18] are used as monotherapy. Thus, it is required to maximize the therapeutic efficacy through drug combination that synergize with TRAIL-based agents. It has been shown that some HDACIs such as Butyrate may increase the expression of DR5 through Sp1 in colon cancer cells [19]. However, it is unclear whether there are other mechanisms at work as well.

Stress response gene ATF3 is a member of the ATF/CREB family. Its messenger RNA (mRNA) level is low or undetectable, but is greatly induced upon exposure of cells to a variety of stress signals including DNA damage, oxidative stress, and cytokines and growth factors [20]. We have previously shown that ATF3 plays a role in p53-dependent DR5 induction upon DNA damage of human colorectal cancer cells [21]. More recently, Wilson et al. have reported that HDACIs sensitized colon cancer cells to apoptosis by a Sp1/Sp3-dependent transcriptional program [22]. However, it still remains elusive whether ATF3 plays role in the transcriptional networks or cell death provoked by HDACIs in human colon cancer cells.

The present study reports that at least six structurally different HDAC inhibitors caused ER stress and activated the PERK-eIF2α pathway of the UPR, leading to up-regulation of DR5 expression on the cell surface of p53-deficient human colon cancer cells. Combined treatment with HDACIs and anti-DR5 monoclonal antibody synergistically sensitized cancer cells to apoptotic cell death. Functional role of ATF3 in HDACIs-induced DR5 gene transcription and cell death was investigated.

2. Materials and methods

2.1. Plasmids, antibodies, and reagents

Retroviral expression plasmid pMX-ATF3 encoding human ATF3 gene was constructed by subcloning cDNA of human ATF3 into pMXs-puro provided by Dr. T Kitamura (University of Tokyo, Japan). Antibodies: anti-KDEL from SressGen (Glanford, Victoria, Canada), anti-phosphorylated PERK (Thr981), anti-PERK (C16), anti-eIF2α (FL315), anti-ATF3 (C19), anti-ATF4 (C20), anti-CHOP (R20) and anti-DR5 (N19) from Santa Cruz (Santa Cruz, CA, USA), anti-β-actin (A2228) from Sigma–Aldrich (St. Louis, MO, USA), anti-PARP from R&D systems (Minneapolis, MN, USA), anti-phosphorylated eIF2α, anti-cleaved caspase-3 from Cell Signaling Technology (Beverly, MA, USA). Sodium butyrate was purchased from Sigma–Aldrich (St. Louis, MO, USA), MS-275, M344 and TSA were purchased from Selleckchem (Houston, TX, USA), SAHA was from Cayman chemical (Ann Arbor, MI, USA), VPA was from TORICS (Minneapolis, MN, USA), DJR2-2 was produced as described previously [23] and normal IgG was purchased from Santa Cruz (Santa Cruz, CA, USA). Other chemicals were reagents grade.

2.2. Cell lines and cell culture

Human colorectal carcinoma HCT116 p53 null cells were provided from Dr. Vogelstein and cultured in McCoy medium supplemented with 10% FBS and 1% P/S. SW480 and HT29 cells were obtained from American Type Culture Collection (Manassas, VA, USA) and cultured in DMEM supplemented with 10% FBS and 1% P/S.

2.3. Cell extracts preparation and Western blot analysis

Cells treated as indicated were harvested and washed in PBS. Whole cell extracts were separated by SDS–PAGE and subjected to Western blot analysis as described [21]. Protein bands were visualized by ECL and captured by image capture with Image Quant Las 500 (General Electric Company, USA). β-Actin was used as a loading control.

2.4. Knockdown of ATF3 and ATF4

ATF3 targeting shRNAs 363, 5′-TGGAAAGTGTGTGAATGCTGAACT-3′; 493, 5′-AAGAACGAGAAGCAGCATTTGATAT-3′; 500, 5′-GAAGATGAGAGAAACCTCTTTATCCAACAGA-3′ were cloned into the retrovirus vector, pMX-puroII-U6, and transfected into Plat E cells to generate retroviruses [24]. HCT116 and SW480 cells stably expressing ecotropic retrovirus receptor mCAT were infected with a mixture of these retroviruses and selected by puromycin to obtain ATF3 knocked-down cells. For silencing ATF4, siRNA oligos specific for ATF4 (sc-35112) and control siRNA were purchased from Santa Cruz or Qiagen, respectively. Transfection of each siRNA was performed using X-tremeGENE siRNA transfection reagent (Roche Applied Science, Basel, Switzerland) according to the manufacturer's instructions.

2.5. Quantitative RT-PCR

Quantitative RT-PCR was performed as described [21]. Primer: ATF4, 5′-TGAAGGAGTTCGACTTGGATGCC-3′ (forward) and 5′-CAGAAGGTCATCTGGCATGGTTTC-3′ (reverse); ATF3, 5′-CTCCTGGGTCACTGGTGTT-3′ (forward) and 5′-TCTGAGCCTTCAGTTCAGCA-3′ (reverse); DR5, 5′-CAGGTGTCAACATGTTGTCC-3′ (forward) and 5′-ATCGAAGCACTGTCTCAGAG-30 (reverse); CHOP, 5′-ATCTGCTTTCAGGTGTGGTG-3′ (forward) and 5′-AGAGCCAAAATCAGAGCTGG-3′ (reverse); GAPDH, 5′-GAGTCAACGGATTTGGTCGT-3′ (forward) and 5′-TTGATTTTGGAGGGATCTCG-3′ (reverse); GAPDH was used as an internal control. Data represent means with S.E. bars of three independent experiments.

2.6. Assessment of cell-surface DR5 by FACS

Cells (2.0×10^6) were treated with HDACIs or DMSO for 24 h, and resuspended in ice-cold FACS buffer of 1% BSA and 0.1% NaN_3 in PBS. Cells were then incubated on ice for 30 min with DJR2-2 or IgG1 isotype control (e-Bioscience, USA). After further incubation with 10 μg/ml streptavidin-PE (Cappel, Aurora, OH) or control IgG-PE (Nacalai tesque, Japan), cells were analyzed by flow cytometry.

2.7. Luciferase assay

Various 5′-deletions of human DR5 gene reporter plasmids and pDR5Luc-552 plasmids containing CHOP, Elk-1, and NF-κB site mutants were gifts from Dr. T Sakai (Kyoto Prefectural University, Japan) and Dr. HG Wang (H. Lee Moffitt Cancer Center, USA), respectively. A set of pDR5Luc-448 reporter plasmids containing mutation of ATF3-2, -3, -4 or -3/4 sites were prepared by an overlap extension PCR protocol. Each of human DR5 gene reporter plasmids was transfected into cells and luciferase activity was measured using a Dual Luciferase Reporter Assay System from Promega as described [21]. Data represent means with S.E. bars of three independent experiments. Concentration of protein and renilla Luciferase activity were used as an internal control.

2.8. Cell death assay

Cell death was estimated by trypan blue exclusion assay [21]. Relative values of dead cell numbers were showed. Data represent

means with S.E. of three independent experiments. For morphological analysis of nucleus, cells were fixed in methanol, incubated with 4′, 6-diamidino-2-phenylindole, and then analyzed using a fluorescent microscope (Olympus, Tokyo, Japan) at 420 nm.

2.9. Statistical analysis

All results were subjected to statistical analysis using two-way analysis of variance, and, wherever appropriate, analyzed by Student-Newman-Keuls test. Data are expressed as mean ± SD of triplicate samples from at least three independent experiments and values that were $P < 0.05$ were considered statistically significant.

3. Results

3.1. HDACIs cause ER stress and increase the expression of ATF4, ATF3, CHOP and DR5 in p53-deficient human colon cancer cells

To understand the effect of HDACIs on human colon cancer cells, we first investigated the effect of SAHA on the expression of ER stress markers and the downstream genes ATF4, ATF3, CHOP and DR5 in human colon cancer cell lines harboring p53 mutations. As shown in Fig. 1A, SAHA significantly increased the expression of ER chaperon GRP78 and elevated the phosphorylation level of RNA-dependent protein kinase-like ER kinase (PERK) and eukaryotic translation initiation factor 2 on the alpha subunit (eIF2α), and subsequently followed by the increased expression of ATF4, ATF3, CHOP and DR5 proteins in HCT116 p53 null, SW480 and HT29 cells. Fig. 1B shows the results of flow cytometry where we examined the cell surface expression of DR5. SAHA increased the level of DR5 expression on the cell surface of these cells in varying degrees, while the effect was barely observed in HT29 cells. Next, we carried out a quantitative RT-PCR, and data showed that mRNAs of ATF4, ATF3, CHOP and DR5 were up-regulated by SAHA in HCT116 p53 null cells (Fig. 1C). We further examined the effects of other HDACIs. As shown in Fig. 1D, HDACIs including trichostatint A (TSA), M344, MS-275, sodium butyrate (But) and volporic acid (VPA) were demonstrated to activate the expressions of ER stress markers as well as ATF4, ATF3, CHOP and DR5 proteins by Western blot. The cell surface expression of DR5 was also enhanced to a comparable extent by these compounds. These data indicated that HDACIs tested in this study caused the activation of PERK-eIF2α axis of the UPR and induced the expression of ATF4, ATF3, CHOP and DR5 in p53-deficient human colon cancer cells.

3.2. The effect of ATF3 or ATF4 knockdown on human colon cancer cells treated by HDACIs

To elucidate role of ATF3 and ATF4 in HDACIs-induced DR5 expression, we employed a loss of gene function approach using

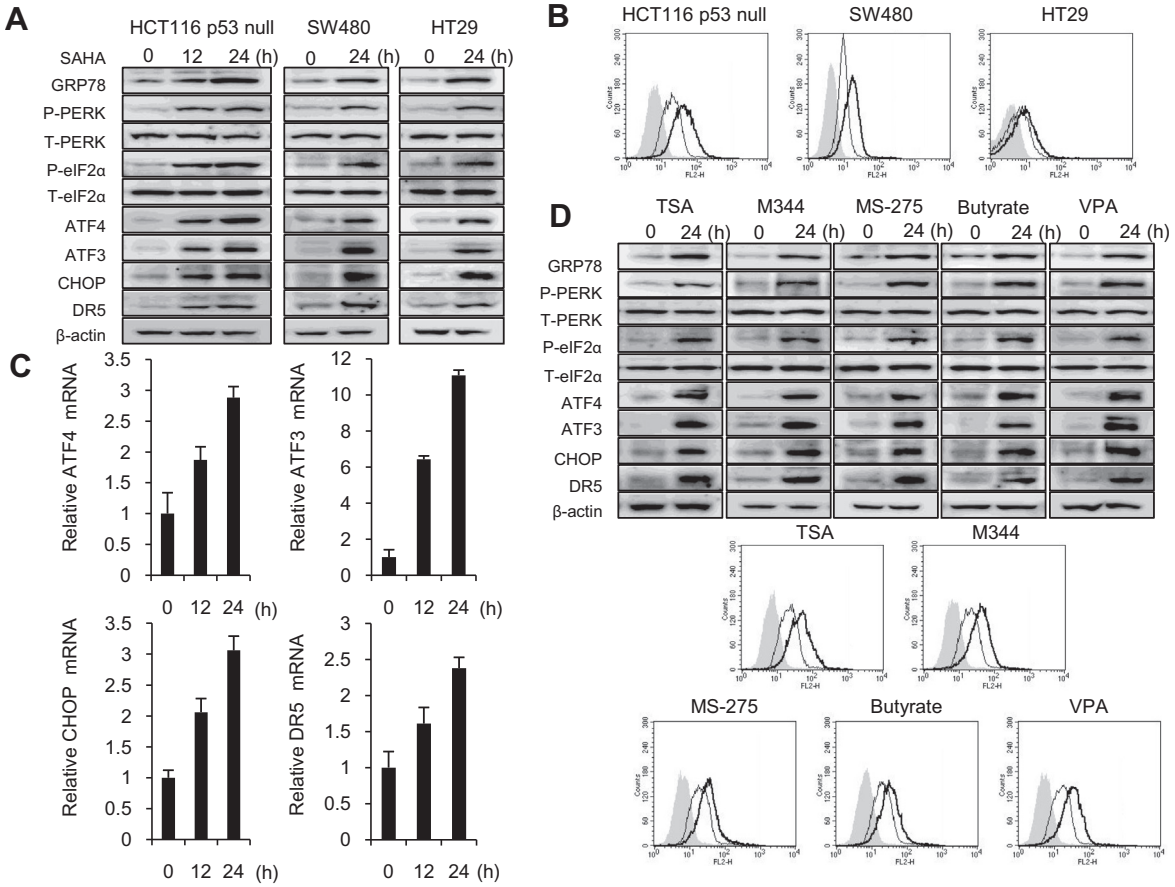

Fig. 1. HDACIs activated the PERK-eIF2α axis of the UPR and upregulate the expression of ER stress markers and ATF4, ATF3, CHOP and DR5. (A) HCT116 p53 null, SW480 and HT29 cells were treated with 5 μM SAHA for the indicated time, and their whole-cell extracts were analyzed for GRP78, PERK-eIF2α axis, ATF4, ATF3, CHOP and DR5 proteins by Western blot. (B) Cell surface expression of DR5 was measured by flow cytometry of the indicated cells following 5 μM SAHA treatment for 24 h using anti-DR5 antibody conjugated to phycoerythrin (shaded no line, DMSO vehicle with isotype-matched control antibody; thin line, DMSO vehicle with anti-DR5 antibody; thick line, HDACIs with anti-DR5 antibody). (C) ATF4, ATF3, CHOP and DR5 mRNA levels of HCT116 p53 null cells as treated in (A) were determined by qRT-PCR. (D) HCT116 p53 null cells were treated with different HDACIs (1 μM TSA, 5 μM M344, 1 μM MS-275, 5 mM But, 10 mM VPA) for 24 h, and their expression of GRP78, PERK-eIF2α axis and ATF4, ATF3, CHOP and DR5 proteins (upper) and cell surface expression of DR5 (lower) were determined as in (A and B), respectively.

siRNAs specific for ATF3 or ATF4. It was observed that the knockdown of ATF3 suppressed the induction of DR5 protein and mRNA, but had no apparent effect on the induction of ATF4 and CHOP (Fig. 2A). By contrast, the ATF4 knockdown suppressed the induction of ATF3, CHOP and DR5 proteins and mRNAs in human colon cancer cells (Fig. 2B). We further examined the effect of ATF3 or ATF4 knockdown in cells treated by other HDACIs. As in Fig. 2C, knockdown of ATF3 suppressed the induction of DR5 protein and knockdown of ATF4 suppressed the induction of ATF3, CHOP and DR5 proteins by these HDACIs. These results suggest that ATF3 plays a role in induction of DR5 by HDACIs in human colon cancer cells and ATF4 is an upstream gene of ATF3, CHOP, and DR5.

3.3. ATF3 and CHOP are required for the efficient activation of the human DR5 gene promoter in response to SAHA

Data above strongly support that ATF3 activates the DR5 gene transcription upon HDACI treatment. To decipher which binding site (BS) on the DR5 gene promoter (Fig. 3A) plays role(s) in HDAC-I-induced activation of the DR5 gene, we assayed the promoter activity of the human DR5 gene using various luciferase reporter constructs. Fig. 3B shows that pDR5-2.5 k, -1.2 k, -552, -448 and -418-Luc reporters, but not further deletion to −198 and −168, were clearly activated by SAHA, demonstrating that SAHA response elements are located between −448 to −198 or −168 that contains notable elements of ATF3-BS2, -3, -4, CHOP, Elk-1 and NF-κB. Thus, we measured the activity of reporters with mutations of Elk-1, NF-κB, or CHOP. As shown in Fig. 3C, Elk-1 or NF-κB site mutants had no significant effect. By contrast, the mutation of CHOP binding site caused significant reduction, indicating the CHOP element was involved in the SAHA-induced activation of DR5 promoter. Next, we constructed a set of mutation of ATF3-BS2, -BS3, -BS4 or triple mutation of the pDR5-448-Luc reporter. As shown in Fig. 3D, BS2 mutant alone did not cause effect, but BS3, BS4 or triple mutant significantly abrogated the induction, indicating both ATF3-BS3 and BS4 were required for the efficient SAHA-induced activation of DR5 promoter. Collectively, these data support that both ATF3 and CHOP binding sites are required for the SAHA-induced DR5 gene activation.

3.4. ATF3 is required for sensitizing p53-deficient cancer cells to apoptosis by a combination of SAHA and anti-DR5 agonistic antibody

It is hypothesized that HDACIs might enhance DR5-mediated apoptosis due to their ability to increase the cell surface expression of DR5 in cancer cells. Thus, we next analyzed cell death by SAHA and anti-DR5 agonistic antibody DJR2-2 in HCT116 p53 null cells. As shown in Fig. 4A, SAHA (0.5 μM) or DJR2-2 (2.5 μg/ml) alone caused 14.6% or 12.4% apoptosis at 24 h, respectively. However, cell death was synergistically increased to 53.9% at 24 h with a combination of these reagents (Fig. 4B). This increase of cell death was significantly suppressed in ATF3 knocked-down cells (Fig. 4B),

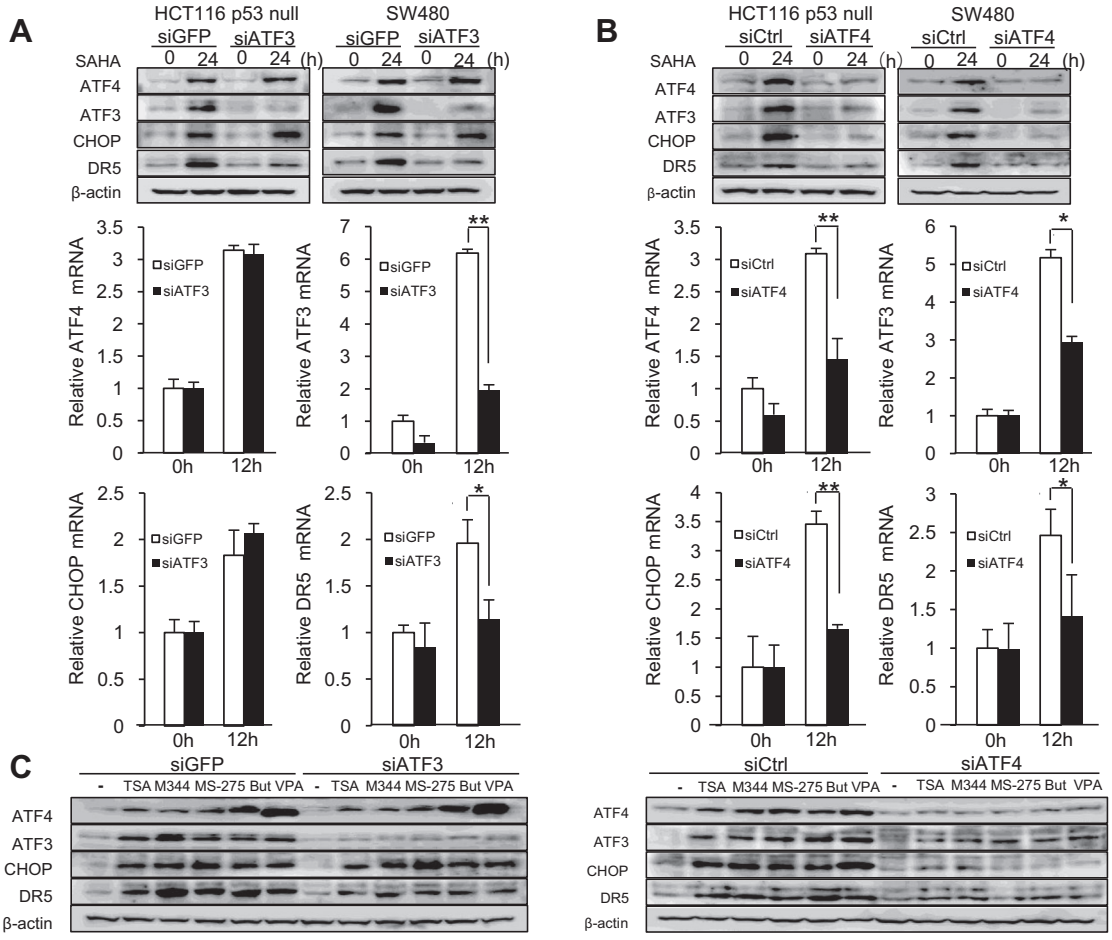

Fig. 2. The effect of ATF3 or ATF4 knockdown to human colon cancer cells treated by HDACIs. ATF3 or ATF4 were knocked down as in Methods. (A) Cells of ATF3 knockdown were treated with 5 μM SAHA for the indicated time and analyzed for the expression of ATF4, ATF3, CHOP and DR5 proteins and mRNA. (B) Cells of ATF4 knockdown were treated with 5 μM SAHA for the indicated time and analyzed for the expression of ATF4, ATF3, CHOP and DR5 proteins and mRNA.*$P < 0.05$; **$P < 0.01$. (C) HCT116 p53 null cells were treated with different HDACIs as in Fig. 1D. ATF4, ATF3, CHOP and DR5 proteins were measured by Western blot.

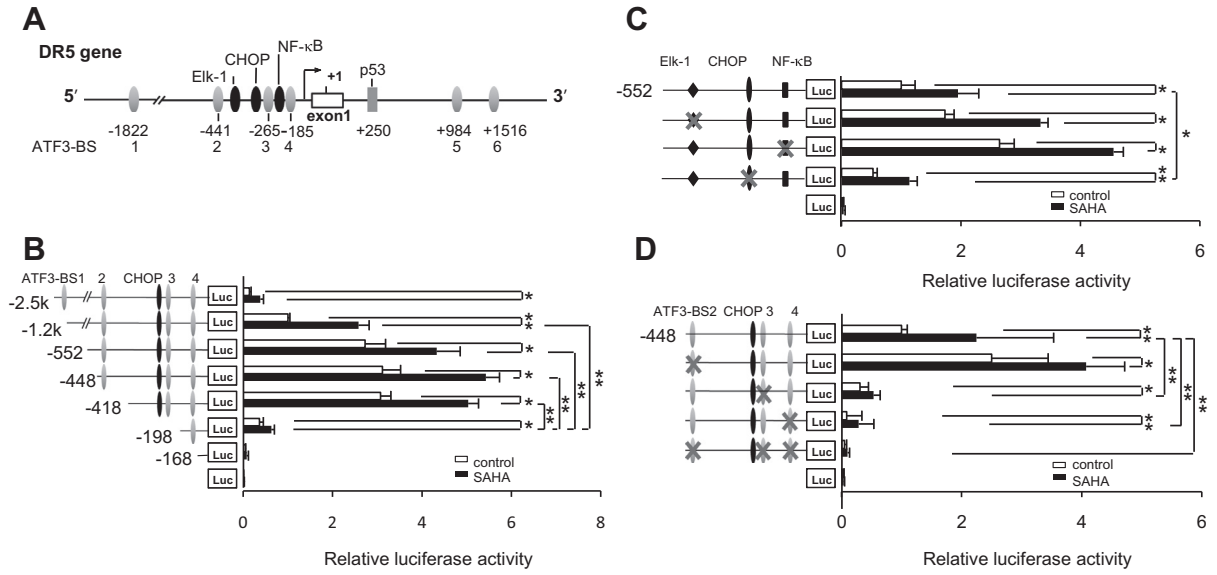

Fig. 3. ATF3 and CHOP binding sites were required for the activation of the human DR5 gene reporter in response to SAHA. (A) Schematic representation of the human DR5 gene promoter. Binding sites for ATF3/CREB (ATF3-BS1 through 6), CHOP, Elk-1, NF-κB and p53 are shown. (B) HCT116 p53 null cells were transfected with 0.5 μg each of pDR5-2.5 k or various 5′-deletions. After 16 h, cells were treated with 5 μM SAHA for another 12 h, and then cell extracts were assayed for luciferase activity. Relative luciferase activity represents the fold induction compared to that of non-stimulated cells transfected with pDR5-1.2 k. (C) HCT116 p53 null cells were transfected with 0.5 μg each of pDR5-Luc552 reporters containing mutations at CHOP, Elk-1 or NF-κB sites as indicated and treated as in (B). Relative luciferase activity is the fold induction compared with that of untreated pDR5-Luc552 reporter. (D) HCT116 p53 null cells were transfected with 0.5 μg each of pDR5-Luc448 reporters containing mutations at ATF3-BS2, -BS3, -BS4 or triple ATF3-BS2/BS3/BS4 sites and treated as in (B). Relative luciferase activity represents the fold induction compared with that of untreated pDR5-Luc448 reporter. All the data are shown as means with S.E. bars of three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$.

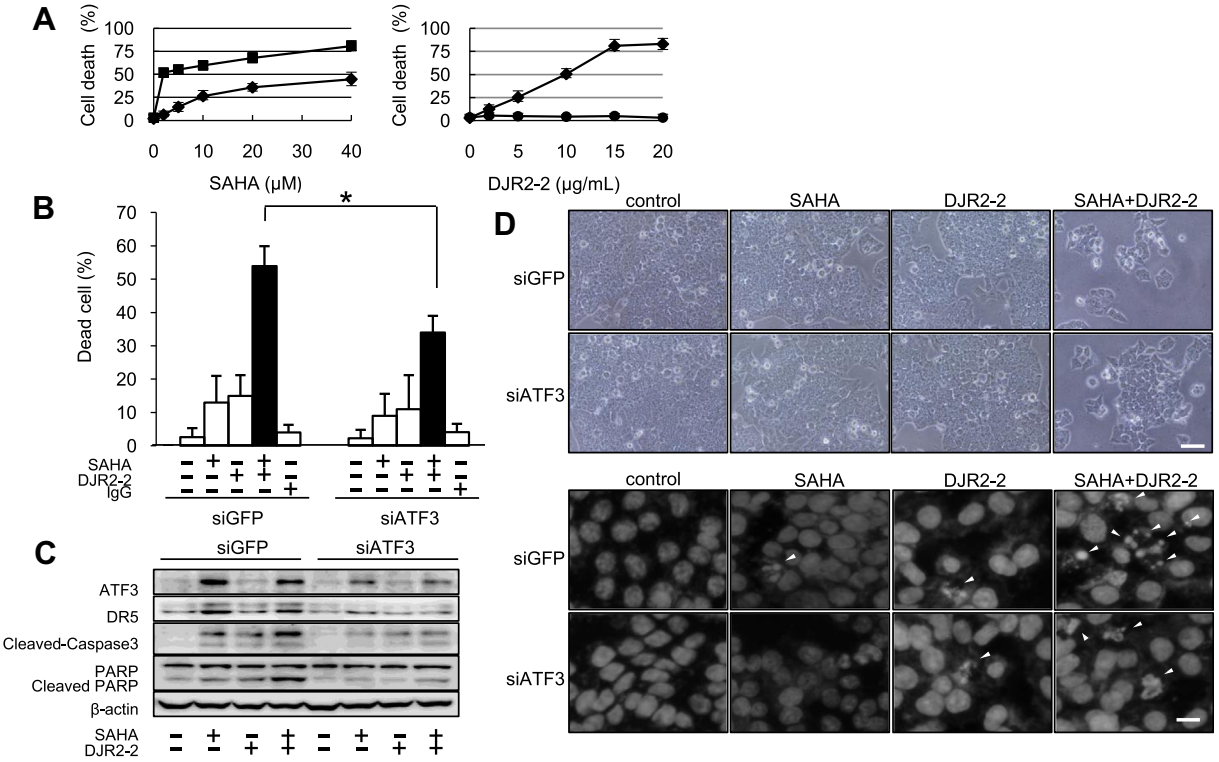

Fig. 4. SAHA and DJR2-2 enhanced DR5-mediated cell apoptosis in an ATF3-dependent manner. (A) HCT116 p53 null cells were incubated with increasing concentrations of SAHA (left) or DJR2-2 (right) for 24 h (diamonds) or 48 h (squares). Mouse IgG (circles) was used as control for DJR2-2. Date represent means with S.E. of three independent experiments. (B) ATF3 knocked-down HCT116 p53 null cells were treated with 5 μM SAHA with or without 2.5 μg/ml DJR2-2 for 24 h. Cell death was examined by trypan blue exclusion assay. Relative values of dead cell numbers are shown and represent means with S.E. of three independent experiments. $^{*}P < 0.05$ (C). ATF3 knocked-down HCT116 p53 null cells were treated with 5 μM SAHA with or without 2.5 μg/ml DJR2-2 for 24 h, and then ATF3, DR5, cleaved-caspase3 and PARP proteins were analyzed by Western blot. (D) ATF3 was knocked down in HCT116 p53 null cells and treated as in (B). Images of cells are shown (upper, scale bar: 50 μm). Nuclei of cells were also visualized with 4′, 6-diamidino-2-phenylindole staining under a fluorescence microscope (lower, scale bar: 20 μm). Arrowheads indicate apoptotic cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and this suppression was accompanied by a biochemical evidence that the cleavage of PARP and caspase-3 by SAHA and DJR2-2 was significantly inhibited in these cells (Fig. 4C). Microscopic study showed that the number of cells with apoptotic property of nuclear condensation or fragmentation decreased in ATF3-knocked down cells (Fig. 4D). Accordingly, it is indicated that the combination treatment with SAHA and DJR2-2 sensitized cancer cells to apoptosis, and this was at least in part mediated via ATF3-dependent expression of DR5 on the cell surface of cancer cells.

4. Discussion

In this study, we have clearly demonstrated that HDACIs including SAHA, TSA, M344, MS-275, But and VPA, all caused the induction of GRP78 and the activation of PERK in ER in human p53-deficient colorectal cancer cells. This is in part consistent with previous reports in which HDACI activated the PERK-eIF2α [4], and supports that HDACIs cause the accumulation of misfolded or unfolded proteins in the ER. It should be noted that six different HDACIs used in this study belong to three structurally categorized groups. SAHA, TSA and M344 belong to hydroxamic acids, VPA and But to short chain fatty acids, and MS-275 to benzamides, respectively [8]. In the present study, it was not addressed what mechanism(s) is responsible for HDACIs to induce the ER stress. However, it is of intrigue to note that some reports have demonstrated that HDACIs induce ER stress through hyper-acetylation of GRP78 [4,5]. Thus, it is likely that HDACIs affected acetylation/dysacetylation of ER-resident chaperons to activate the UPR. Alternatively, generation of reactive oxygen species (ROS) may underlie the ER stress by HDACIs since several reports demonstrated that ROS may be important in HDACIs-induced apoptosis of cancer cells [25,26], and protein folding and generation of ROS in the ER are closely linked events [27]. However, the detailed mechanism must await further study.

The present study showed that HDACIs activated the PERK-eIF2α branch of the UPR and induced its downstream gene transcriptional program of ATF4/ATF3/CHOP pathway. Consistent with this, ATF3 was induced downstream of ATF4 in HDACIs-treated cancer cells, and it played significant role in the HDACIs-induced expression of DR5 along with CHOP. The functional role of ATF3 was further supported by our experiment where *Atf3−/−/p53−/−* mouse embryonic fibroblasts were treated by HDACIs. DR5 induction was significantly suppressed in these cells, but rescued by re-introduction of ATF3 (Supplementary Fig. 1). Thus, it is likely that these two transcription factors play crucial role in the transcriptional activation of the *DR5* gene by HDACIs, possibly in a co-operative manner.

In this study, co-treatment of SAHA and DJR2-2 synergistically increased cancer cell death. Our findings that HDACIs induced the cell surface expression of DR5 strongly support the idea that the induction of DR5 by HDACIs could be a key factor to maximize the efficacy of DR5 agonist antibody for sensitizing HCT116 p53 null cells to apoptosis. In this regard, the induction of cell-surface expression of DR5 in HT29 cells was less than those in HCT116 or SW480 cells. This might due to improper transport of DR5 to the cell surface [28], and correlated with previous report that HT29 cell line is defined as being resistant to HDACIs while HCT116 and SW480 cells are sensitive [22].

Collectively, our present work demonstrates that HDACIs cause the ER stress and sensitizes human colon cancer cells to apoptosis by DJR2-2. ATF3 and CHOP are implicated as pro-apoptotic transcription factors to activate the DR5 gene transcription. Particularly, the role of ATF3 provide important mechanistic insight into using HDACIs as anti-cancer agents in future clinical scenarios when they may be used alone or in combination with other therapeutic strategies of cancer.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.01.184.

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