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Implication of Nrf2 and ATF4 in differential induction of CHOP by proteasome inhibition in thyroid cancer cells

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ABSTRACT

Proteasome inhibition may cause endoplasmic reticulum (ER) stress, which has been reported to be implicated in the antitumoral effects of proteasome inhibitors. CCAAT/enhancer-binding protein homologous protein (CHOP) is induced by a variety of adverse physiological conditions including ER stress and is involved in apoptosis. We have reported that distinct induction of CHOP contributes to the responsiveness of thyroid cancer cells to proteasome inhibitors. However, the mechanism underlying differential induction of CHOP by proteasome inhibitors in thyroid cancer cells has not been well characterized. In the current study, we characterized that proteasome inhibition primarily activated the amino acid response element 1 (AARE1) on the CHOP promoter. We also demonstrated that although proteasome inhibition caused similar accumulation of activating transcription factor 4 (ATF4) in a panel of thyroid cancer cells, distinct amounts of ATF4 were recruited to the AARE1 element of CHOP promoter. In addition, we demonstrated that NF-E2-related factor 2 (Nrf2) was also implicated in the induction of CHOP by precluding the binding of ATF4 to the CHOP promoter. This study highlights the molecular mechanisms by which ATF4 and Nrf2 can control CHOP induction in thyroid cancer cells by proteasome inhibition.

1. Introduction

Proteasome inhibitors possess the pre-clinical and clinical activities against hematologic malignancies and solid tumors [1]. The inhibition of the 26S proteasome may lead to the accumulation and aggregation of misfolded proteins in the endoplasmic reticulum (ER) lumen, subsequently cause ER stress. In response to ER stress, activating transcription factor 6 (ATF6), inositol requiring 1 (Ire1) and PKR-like endoplasmic reticulum kinase (PERK), along with other proximal signaling molecules, initiate a program of transcriptional and translational regulation termed the unfolded protein response (UPR). Accumulating evidence now supports that, in addition to mitochondria, the ER also serves as an important intracellular apoptotic control point. The ER-mediated apoptotic pathway is triggered by ER stress, which leads to proapoptotic UPR including induction of CHOP, activation of the apoptosis signal-regulating kinase 1 (ASK1)-c-Jun-N-terminal kinase (JNK) pathway and cleavage of ER resident caspases including caspase 12 (in rodent) and caspase 4 (in human) [2]. It has been widely reported that ER stress is implicated in antitumoral effects of proteasome inhibitors [3–7].

CHOP, also known as GADD153, is a member of the C/EBP family transcription factor family that heterodimerizes with other C/EBPs [8]. Its basal expression is very low under nonstressed conditions, but it is induced by a variety of adverse physiological conditions [9,10]. Several studies point to a proapoptotic effect of CHOP downstream of irremediable ER stress [11–14]. Regulation of CHOP expression is cell and stimulus dependent, which consequently affects the outcome of ER stress [15–18]. We have previously shown that CHOP-mediated apoptosis is involved in the cytotoxicity induced by proteasome inhibitors in thyroid cancer cells [7]. In a panel of thyroid cancer cells, proteasome inhibitors increase the expression of GRP78/Bip with similar extents, but CHOP is differently induced and the differential induction of CHOP appears to be implicated in the responsiveness [7]. However, the mechanism(s) underlying preferential induction of CHOP in sensitive thyroid cancer cells compared with those insensitive cells remains unclear. The CHOP gene can be activated through the ER stress response elements (ERSE, bases −103 to −76) in response to cellular stress [19], amino acid response elements (AARE, bases −310 to 302 and base −778 to −770) in response to amino acid starvation [15,20,21], and C/EBP-ATF composite site, a part of AARE1, in response to ER stress [18]. In addition, it can also be activated through activator protein 1 (AP-1, bases −244 to −238) element in response to oxidative stress and in mitochondrial unfolded protein response [15,21]. Since ERSEs are also present in the promoters of other unfolded protein response target genes, including GRP78/BiP [22], coupled with the fact that it has similar extent of upregulation of GRP78

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in the panel of thyroid cancer cells treated with proteasome inhibitors [7], proteasome inhibitors might induce CHOP expression via AARE and/or AP-1, but not via ERSE.

It is now established that a multiprotein complex is bound to the AARE of CHOP promoter including a number of regulatory proteins such as activating transcription factor 4 (ATF4), C/EBPβ, TRB3, PCAF and ATF2 [17]. ATF4 is a member of the cyclic adenosine monophosphate responsive element-binding (CREB) protein family, which is activated by PERK during UPR and involved in multiple intracellular stress pathways [23]. PERK coordinates the convergence of ER stress with oxidative stress signaling via activation of ATF4 and Nrf2 transcription factors [24]. ATF4 signaling promotes two divergent transcriptional programs: increased transcription of both pro-survival and pro-apoptotic genes [24]. On the other hand, Nrf2 signaling in response to ER stress is cytoprotective [25,26]. The target genes for Nrf2 and ATF4 are somewhat overlapping [27–29], for example, both Nrf2 and ATF4 are implicated in the regulation of CHOP, Nrf2 inhibits, while ATF4 promotes CHOP expression [18,25,30]. In the current study, we find that proteasome inhibition primarily activates CHOP expression via ATF4 binding to the AARE1 element of CHOP promoter, while Nrf2 is implicated in differential induction of CHOP in the panel of thyroid cancer cells by precluding the recruitment of ATF4 to the AARE1 element of CHOP promoter.

2. Materials and methods

2.1. Culture of multiple cancer cell lines

FRO82-1 (simply FRO) cell lines were initially obtained from Dr. James A. Fagin (University of Cincinnati College of Medicine, Cincinnati, OH) and provided to us by Dr. Shunichi Yamashita (Nagasaki University Graduate School of Biomedical Sciences, Japan). KTC1 and KTC3 cell lines were generously provided by Dr. Junichi Kurebayashi (Kawasaki Medical School, Japan). 8305C and 8505C cells were obtained from the European Collection of Animal Cell Cultures. The earliest passage of each cell line received in our laboratories was DNA profiled using the Applied Biosystems Profiler Plus kit (ABI, Foster, CA). Consistent with the previous report [31], the STR profiles of all these cell lines were consistent with their respective profiles in the DSMZ database (http://www.dsmz.de/). All cell lines were maintained in DMEM (Sigma-Aldrich, Saint Louis, MO) supplemented with 10% fetal bovine serum (FBS, ExCell Biology Inc., Shanghai, China).

2.2. Chemicals

MG132, SB203580 and SP600125 were purchased from Calbiochem (La Jolla, CA). Bortezomib was obtained from Millennium Pharmaceuticals Inc. (Cambridge, MA). Specific GSH synthesis inhibitor (BSO) and N-acetyl-L-cysteine (NAC) were from Sigma-Aldrich and Calbiochem (San Diego, CA), respectively. 0.02% DMSO was used as vehicle control.

2.3. RNA isolation and real-time reverse transcription-polymerase chain reaction (RT-PCR)

RNA isolation and real-time RT-PCR were performed as previously reported [7]. For ATF4, the forward primer was 5′-TGACCTGGAAACCATGCCAG-3′ and 5′-AATGATCTGGAGTGGAGGAC-3′, the amplicon size was 221 bp. For CHOP, the forward primer was 5′-ATGAGGACCTGCAAGAGGTCC-3′ and the reverse was 5′-TCCTCCTCAGTCAGCCAAGC-3′, the amplicon size was 136 bp. For β-actin, the forward primer was 5′-GAGACCTTCAACACCCCAGCC-3′ and the reverse was 5′-GGATCTTCATGAGGTAGTCAG-3′, the amplicon size was 205 bp. Results were normalized against those of β-actin and presented as arbitrary unit.

2.4. Measurement of intracellular ROS levels

The average level of intracellular ROS was evaluated in cells loaded with the redox-sensitive dye DCFH-DA (Molecular Probes, OR). Cells were washed twice in a phosphate-buffered saline (PBS) and stained in the dark for 30 min with 20 μM DCFH-DA and harvested. Cells were dissolved with 1% Triton X-100, and fluorescence was measured at an excitation wavelength of 485 nm and an emission wavelength using a fluorescence spectrometer (HTS 7000, Perkin Elmer, Boston, MA). A duplicate culture with the same treatments was used to determine the total protein levels. The ROS levels were expressed as arbitrary unit/mg protein, then as the percentage of control.

2.5. Western blot analysis and immunoprecipitation

Cells were lysed in lysis buffer (20 mM Tris–HCl, 150 mM NaCl, 2 mM EDTA, 1% Triton-X 100) and protease inhibitor cocktail (Sigma-Aldrich, Saint Louis, MO). Cell extract protein amounts were quantified using the BCA protein assay kit. Equivalent amounts of protein (25 μg) were separated using 12% SDS-PAGE and transferred to PVDF membrane (Millipore Corporation, Billerica, MA). For immunoprecipitation, lysates of cultured cells were pre-cleared with protein A-Sepharose CL-4B (Amersham Biosciences, Uppsala, Sweden) and were then incubated overnight at 4 °C with Flag antibody and protein A-Sepharose. The immunoprecipitates were washed three times with lysis buffer and analyzed by Western blot analysis.

2.6. Chromosomal immunoprecipitation (ChIP) assay

ChIP analysis was performed as described previously [32]. Real-time quantitative PCR was performed using primers specific for human CHOP sequence between −472 and −301 (forward: 5′-AAGAGGCTCACGACCGACTA-3′ and reverse: 5′-ATGATGCAATGTTTGGCAAC-3′) to generate a 172 bp amplification product containing AARE1. The immunoprecipitation/input ratio of the untreated sample was considered as 100% and the immunoprecipitation/input ratio of the MG132 treated sample was expressed as a percentage of the untreated.

2.7. Construction of plasmids and luciferase assay

The enhancer-luciferase reporter plasmids were constructed by inserting sequences of various response elements into the filled-in NheI/BglII sites of pGL3-Promoter Vector (Promega, Madison, WI) via blunt-end ligation. These enhancer sequences were synthesized chemically as double-stranded oligomers: AARE1 (5′-CCAACATTGCATCATCCCCG-3′ and 5′-CGGGGATGATGCAATGTTTGG-3′), AARE2 (5′-AGTAGAGACGGGGTTTCACCAT-3′ and 5′-ATGGTGAAACCCCGTCTCTACT-3′), ERSE (5′-CCAATGCCGGCGTGCCACTTTCTGATTGG-3′ and 5′-CCAATCAGAAAGTGGCACGCCGGCATTGG-3′), and AP-1 consensus (5′-GGCTTGATGAGTCAGCCGGAA-3′ and 5′-TTCCGGCTGACTCATCAAGCG-3′). Cells were transfected with one of the enhancer-luciferase reporter constructs (AARE1, AARE2, AP-1 and ERSE) and pGL4.74 [hRluc/TK] (Renilla luciferase internal control) plasmid (Promega, Madison, WI). The firefly and renilla luciferase activities were determined using the Dual-Luciferase® Reporter Assay System (Promega, Madison, WI), according to the manufacturer's instructions. All transfection experiments were repeated for three times in triplicate. The result was expressed as relative luciferase activity.

2.8. Generation of CHOP promoter luciferase constructs

The 5′-flanking region of human CHOP genomic DNA between −1031 and +13 (+1 represents the transcription start site) was amplified by PCR from FRO genomic DNA and subcloned into the reporter plasmid pGL4 (Promega, Madison, WI). Four plasmids pΔERSE, pΔAP-1,

pΔAARE1 and pΔAARE2 lacking respective enhancer sequence were generated from pCHOP by a polymerase chain reaction-based method.

2.9. Construction of EGFP-Nrf2 plasmids

WT-Nrf2, Nrf2-$^{\Delta}$NLS, Nrf2-$^{\Delta}$NES, and Nrf2-$^{\Delta}$NES/$^{\Delta}$TAD mutants were subcloned into pcDNA3-Flag as previously reported [33,34]. Nrf2 and its mutant fragments were cut from pcDNA3-Flag plasmid and subcloned into the XbaI/XhoI sites of the eukaryotic expression plasmid pcDNA3-EGFP.

2.10. Fluorescent microscopy

For analysis distribution of EGFP-tagged Nrf2 mutants, images of live cells were taken using the Olympus inverted microscope capable of digital epifluorescence imaging.

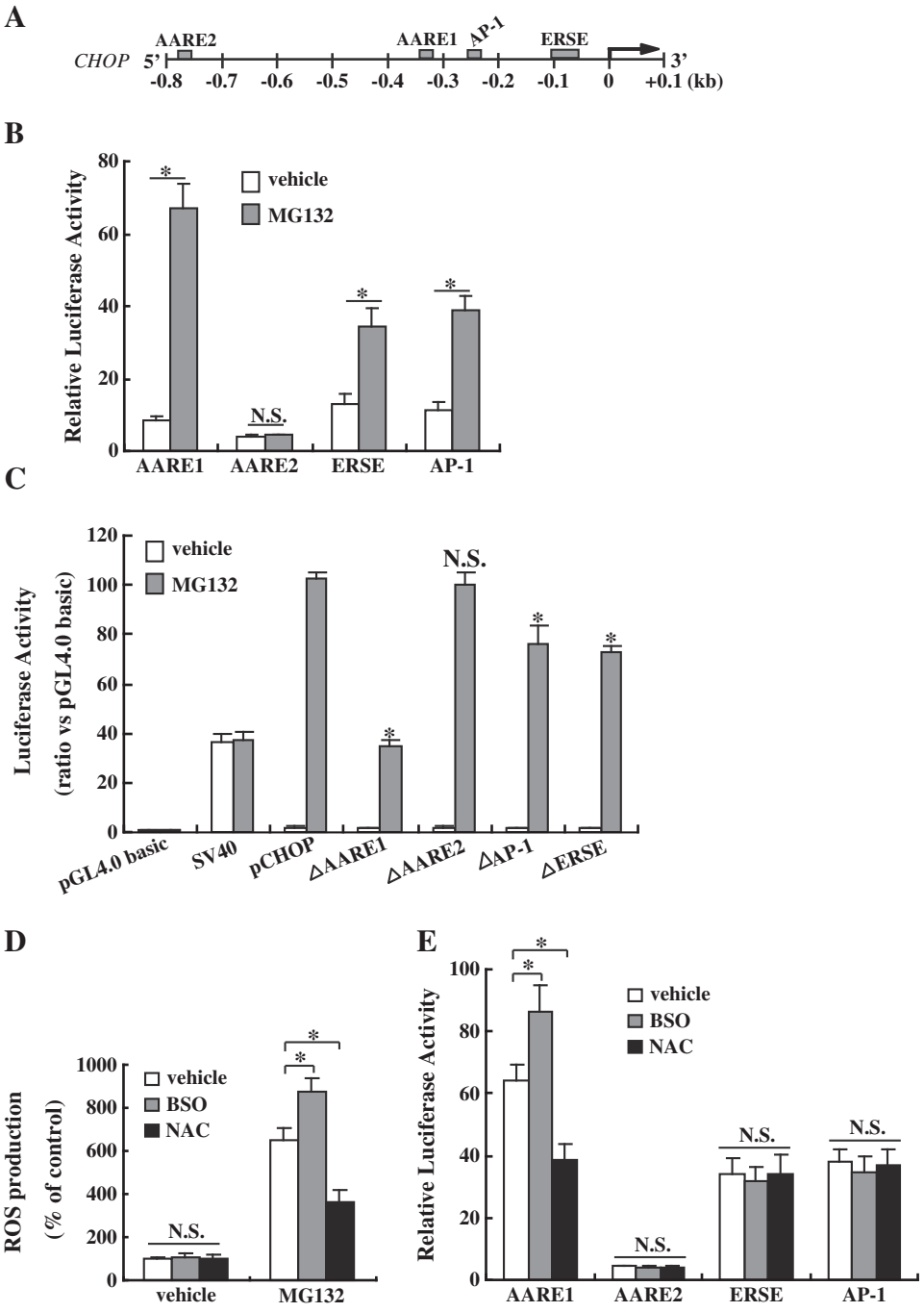

Fig. 1. Effect of MG132 on the promoter activities of CHOP in FRO cells. A, Scheme of the human CHOP gene promoter indicates the different enhancers (boxed in gray) responsible for inducible CHOP expression. B, Cells were transfected with one of the enhancer-luciferase reporter constructs (AARE1, AARE2, ERSE, and AP-1) and pGL4.74 [hRluc/TK] internal control plasmid. 24 h after transfection, the cells were treated with vehicle or 2 μM MG132 for another 8 h, and then firefly and renilla luciferase activities were determined. The results were expressed as relative luciferase activity. C, Cells were transfected with one of the luciferase reporter constructs and pGL4.74 [hRluc/TK] internal control plasmid. 24 h after transfection, the cells were treated with vehicle or 2 μM MG132 for another 8 h, and then firefly and renilla luciferase activities were determined. The results were expressed as ratio vs pGL4.0 basic. D, Cells were pretreated with vehicle, BSO or NAC, sequentially treated with MG132 for additional 8 h, and production of ROS was investigated. E, Cells were transfected with one of enhancer-luciferase reporter constructs (AARE1, AARE2, ERSE, and AP-1) and pGL4.74 [hRluc/TK] internal control plasmid, treated as D and luciferase activities were measured. *, $P<0.01$; N.S., not significant.

2.11. Small interfering RNA (siRNA)

The siRNA sequences used here were as follows: siRNA against Nrf2 (siNrf2), AAGAGUAUGAGCUGGAAAAAC; siRNA against ATF4 (siATF4), CCAGAUCAUUCCUUUAGUUUA; and siRNA against PERK (siPERK), GCAUGCAGUCUCAGACCCATT. The scramble nonsense siRNA (scramble; CCGUAUCGUAAGCAGUACU) that has no homology to any known genes was used as control. Transfection of siRNA oligonucleotide was performed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations.

2.12. Statistics

The statistical significance of the difference was analyzed by ANOVA and post hoc Dunnett's test. Statistical significance was defined as $p<0.05$. All experiments were repeated three times, and data were expressed as the mean ± SD (standard deviation) from a representative experiment.

3. Results

3.1. Activation of AARE1, ERSE and AP-1 element on the CHOP promoter by MG132

The *CHOP* promoter contains several well-characterized response elements: AARE2 (base −778 to −770), AARE1 (bases −310 to 302), AP-1 element (bases −244 to −238), and two ERSE (bases −103 to −76) in reversed orientations (Fig. 1A) [15,20,21]. Using the enhancer-luciferase reporter constructs, activation of the response elements on the *CHOP* promoter by MG132 in FRO cells was investigated. MG132 activated AARE1, ERSE, and AP-1 elements by 8.3-, 2.7-, and 3.4-fold, respectively, but had no obvious effect on the AARE2 element

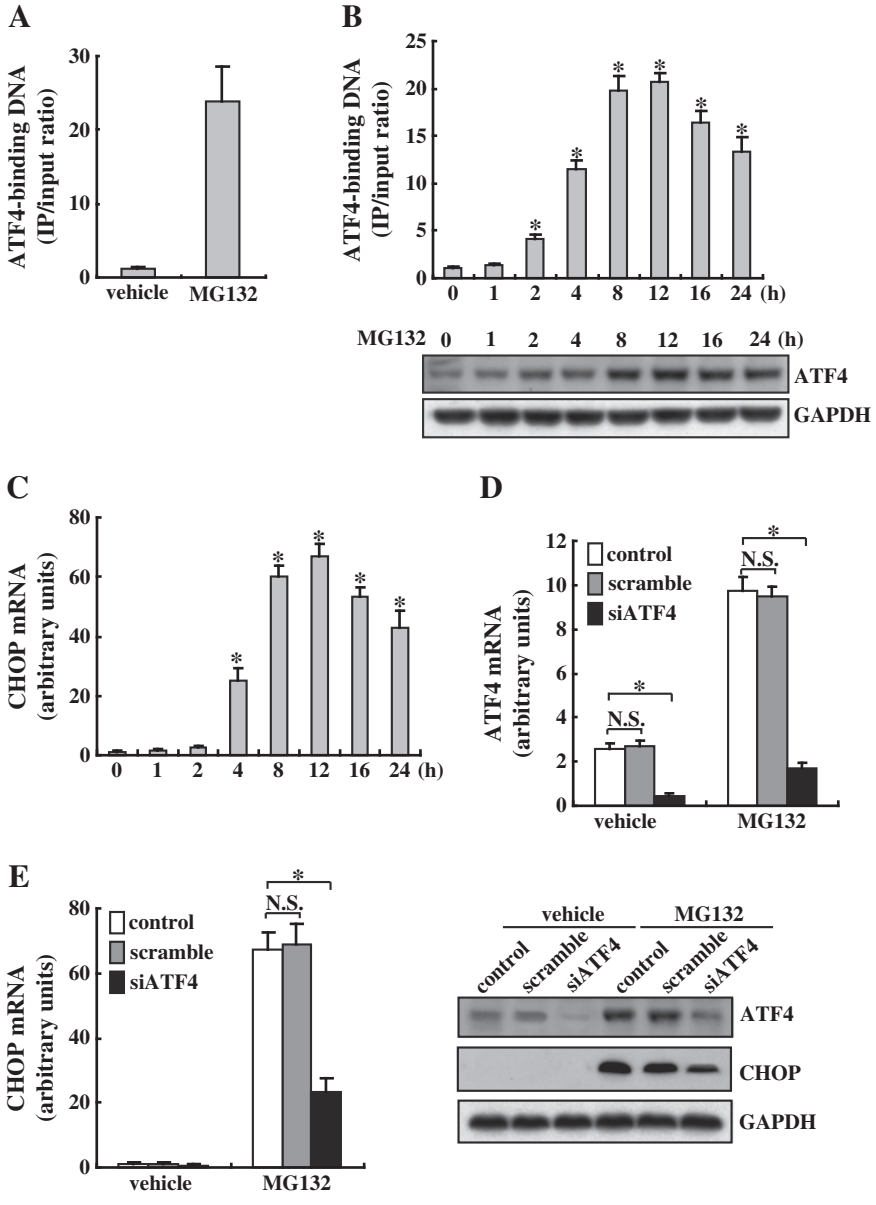

Fig. 2. Recruitment of ATF4 to the AARE1 element on the *CHOP* promoter by MG132. A, FRO cells were exposed to vehicle or 2 μM MG132 for 8 h, ChIP analysis was performed using a specific anti-ATF4 antibody and immunoprecipitated DNA was amplified by real-time PCR. B, FRO cells were treated with 2 μM MG132 for the indicated time, ChIP analysis was performed using a specific anti-ATF4 antibody and immunoprecipitated DNA was amplified by real-time PCR. ATF4 protein levels were confirmed using Western blot and presented under the graph. C, Cells were treated with 2 μM MG132 for the indicated time, real-time RT-PCR was performed. D, FRO cells were transfected with scramble or specific siRNA against ATF4 (siATF4), then treated with 2 μM MG132 for 8 h and ATF4 mRNA was measured using real-time RT-PCR. E, FRO cells were treated as D, and CHOP mRNA and protein were investigated using real-time RT-PCR and Western blot, respectively. *, $P<0.01$; N.S., not significant.

(Fig. 1B). To test the role of these enhancers in CHOP induction by MG132, reporter plasmids containing CHOP promoter with deletion of these enhancer sequences were tested (Fig. 1C). Deletion of AARE1, AP-1 or ERSE partly suppressed MG132-mediated transcriptional induction of CHOP, while deletion of AARE2 demonstrated no obvious effects on transcriptional induction of CHOP by MG132 (Fig. 1C).

To investigate the involvement of oxidative stress in the induction of CHOP by MG132, the effects of BSO (a specific glutathione synthesis inhibitor) and NAC (antioxidant and precursor of glutathione) on the activation of CHOP promoter by MG132 were investigated. Consistent with our previous report [35], pretreatment with 10 mM NAC significantly suppressed MG132-induced reactive oxygen species (ROS) generation, while 0.2 mM BSO pretreatment increased ROS production mediated by MG132 in FRO cells (Fig. 1D). BSO increased and NAC decreased the AARE1 activity, respectively (Fig. 1E). Both BSO and NAC had no obvious effects on AARE2, ERSE or AP-1 activity (Fig. 1E). These results suggested that oxidative stress might be involved in MG132-induced CHOP expression via the AARE1 element.

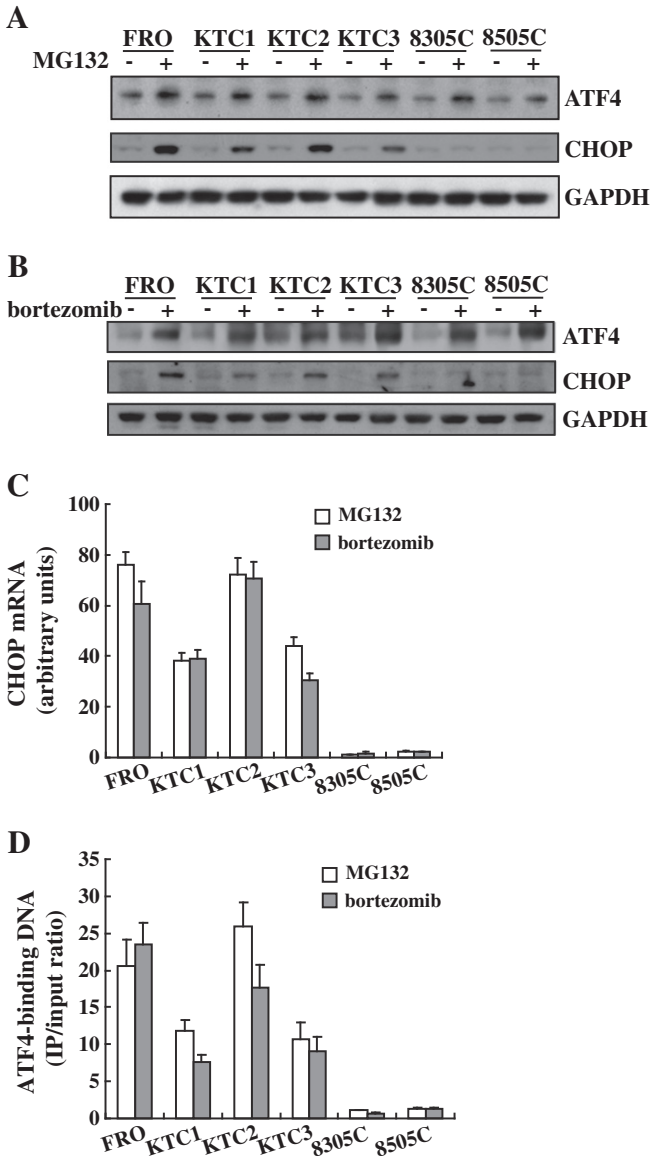

Fig. 3. Involvement of recruitment of ATF4 to the *CHOP* promoter in different induction of CHOP by MG132. A, The panel of thyroid cancer cells were treated with 2 μM MG132 for 8 h, and Western blot analysis was performed. B, The panel of thyroid cancer cells were treated with 50 nM bortezomib for 8 h, and Western blot analysis was performed. C, Cells were treated with 2 μM MG132 or 50 nM bortezomib for 8 h, and CHOP mRNA was measured using real-time RT-PCR. D, Cells were treated with 2 μM MG132 or 50 nM bortezomib for 8 h, ChIP analysis was performed using a specific anti-ATF4 antibody and immunoprecipitated DNA was amplified by real-time PCR.

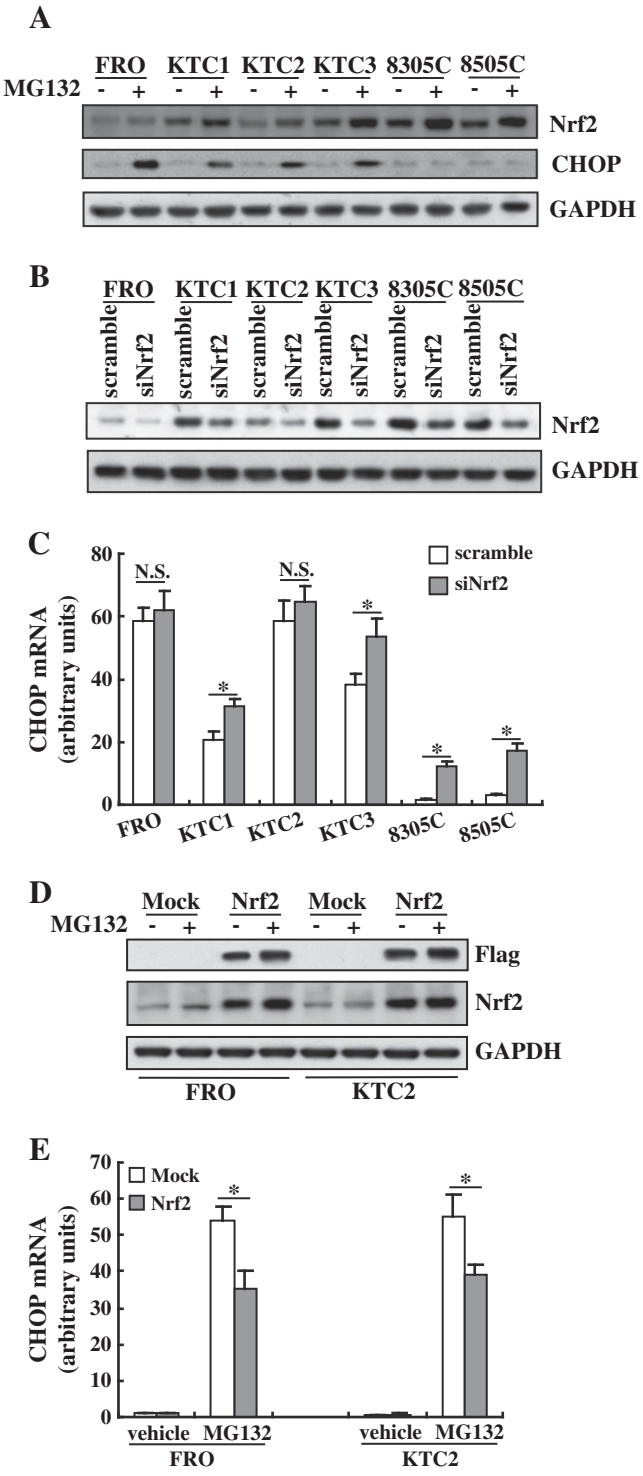

Fig. 4. Involvement of Nrf2 in MG132-mediated induction of CHOP in the thyroid cancer cells. A, The panel of thyroid cancer cells were treated with 2 μM MG132 for 8 h, and Western blot analysis was performed to investigate the expression of Nrf2. B, Cells were transfected with scramble or specific siRNA against Nrf2 (siNrf2), then treated with 2 μM MG132 for 8 h, and Nrf2 expression was measured using Western blot analysis. C, Cells were treated as B, and CHOP mRNA was measured using real-time RT-PCR. D, FRO or KTC2 cells were transfected with mock or Nrf2 eukaryotic expression vector for 24 h, then treated with 2 μM MG132 for 8 h, and Western blot analysis was performed. E, Cells were treated as D, and CHOP mRNA was measured using real-time RT-PCR. *, $P<0.01$.

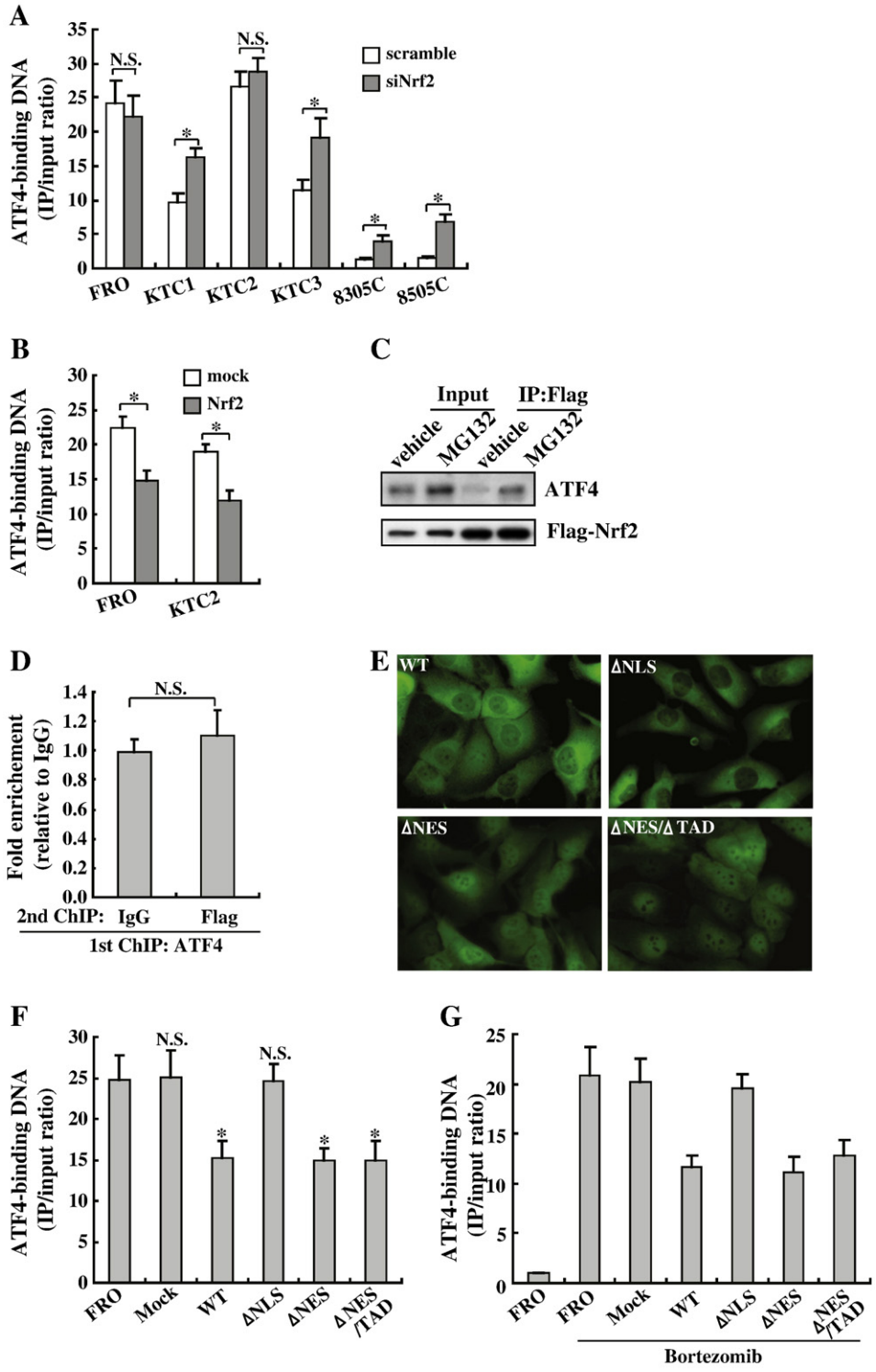

Fig. 5. Regulation of CHOP induction by Nrf2 via modulation of ATF4 recruitment to the CHOP promoter. A, Cells were transfected with scramble or specific siRNA against Nrf2 (siNrf2), then treated with 2 μM MG132 for the indicated time, ChIP analysis was performed using a specific anti-ATF4 antibody and immunoprecipitated DNA was amplified by real-time PCR. B, FRO or KTC2 cells were transfected with mock or Nrf2 eukaryotic expression vector, then treated with 2 μM MG132 for 8 h, ChIP analysis was performed using a specific anti-ATF4 antibody and immunoprecipitated DNA was amplified by real-time PCR. C, FRO cells were transiently transfected with Flag-tagged Nrf2 eukaryotic expression vector, then treated with vehicle or 2 μM MG132 for 8 h, cell lysates (Input) and immunoprecipitates obtained with an anti-Flag antibody (IP) were analyzed by immunoblotting. D, Flag-tagged Nrf2 transfected FRO cells were treated with MG132 for 8 h and re-ChIP assay was performed to assess in vivo colocalization of Nrf2 and ATF4 to the *CHOP* promoter. First ChIP and second ChIP antibodies were anti-ATF4 and anti-Flag, respectively. Quantification of binding was represented as fold change to control IgG second ChIP. E, FRO cells were transfected with EGFP-tagged indicated Nrf2 constructs, and intracellular localization was observed under fluorescent microscopy. F, FRO cells were transfected with the indicated constructs for 24 h and treated with 2 μM MG132 for additional 8 h. ChIP using anti-ATF4 antibody and subsequent real-time PCR were performed to measure enrichment of ATF4 to the *CHOP* promoter by MG132. G, FRO cells were transfected with the indicated constructs for 24 h and treated with 50 nM bortezomib for additional 8 h. ChIP using anti-ATF4 antibody and subsequent real-time PCR were performed to measure enrichment of ATF4 to the *CHOP* promoter by bortezomib. H, FRO cells were transfected with Nrf2 expressing plasmid and siRNA against PERK (siPERK) alone or in combination for 24 h, then treated with vehicle or MG132 for additional 24 h, and Western blot was performed. I, FRO cells were transfected with Nrf2 expressing plasmid and siRNA against PERK (siPERK) alone or in combination for 24 h, then treated with vehicle or MG132 for additional 8 h, and real time PCR was performed. J, FRO cells were cotransfected with EGFP-tagged Nrf2 and scramble or siPERK for 24 h, then treated with vehicle or MG132 for additional 8 h, and localization of EGFP-Nrf2 was observed under a fluorescence microscopy. *, $P<0.01$; N.S., not significant.

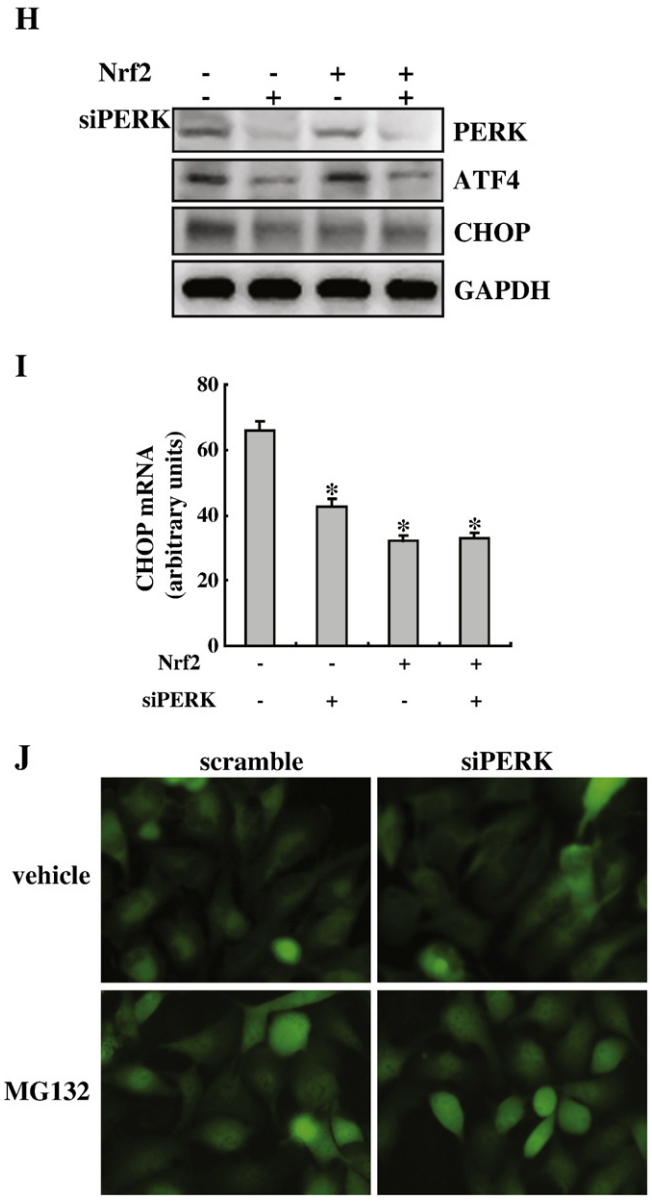

Fig. 5 (*continued*).

3.2. ATF4 binding to the AARE1 element of the CHOP promoter in response to MG132

Since the AARE1 element of the *CHOP* promoter was the main *cis*-acting element involved in the activation of CHOP by MG132 (Fig. 1B), in addition, proteasome inhibitors induced ER stress [3–7,36] and ATF4 was recruited to the AARE1 element to activate CHOP transcription during ER stress [17], we investigated whether ATF4 could be involved in MG132-induced transcription of CHOP. ChIP assays showed that MG132 increased the binding of ATF4 to the AARE1 element of the *CHOP* promoter in FRO cells (Fig. 2A). Kinetic analysis of ATF4 recruitment to the AARE1 element in response to MG132 showed that ATF4 recruitment increased rapidly within 2 h of MG132 exposure, reached a maximal level of binding within 8–12 h and decreased slightly after 16 h (Fig. 2B). Similar to the recruitment of ATF4 to *CHOP* promoter, Western blot confirmed that MG132 caused obvious accumulation of ATF4 protein at 2 h, and reached maximal increase at 8–12 h (Fig. 2B). The CHOP mRNA was clearly increased after 4 h and peaked after 8–12 h (Fig. 2C). The slight delay in CHOP induction indicated that ATF4 might be involved in the induction of CHOP transcription in response to MG132 in FRO cells.

To confirm the implication of ATF4 in proteasome inhibition-mediated CHOP induction, specific siRNA against ATF4 (siATF4) was used to knockdown the expression of ATF4. siATF4 successfully reduced basal as well as MG132-induced ATF4 expression, while scramble siRNA had no effects on ATF4 expression (Fig. 2D). Importantly, siATF4 markedly decreased the induction of CHOP mRNA and protein expression mediated by MG132, while scramble siRNA had no obvious actions (Fig. 2E).

3.3. Different recruitment of ATF4 to the AARE1 element of the CHOP promoter upon proteasome inhibition in a panel of thyroid cancer cells

To investigate the implication of ATF4 in the differential induction of CHOP by proteasome inhibitors in a panel of thyroid cancer cells [7], we firstly studied the expression levels of ATF4 using Western blot and found an accumulation of ATF4 protein by MG132 with similar extents in the panel of thyroid cancer cells (Fig. 3A). Another proteasome inhibitor bortezomib also caused ATF4 accumulation with similar extents in these

cells (Fig. 3B). Consistent with our previous report [7], real-time RT-PCR confirmed that the levels of CHOP mRNA were significantly induced in FRO, KTC1, KTC2 and KTC3, only a slight increase was detected in 8505C cells, whereas no induction of CHOP mRNA was observed in 8305C cells after treatment with MG132 for 8 h (Fig. 3C). Bortezomib demonstrated similar pattern of CHOP induction (Fig. 3C). We further investigated the binding of ATF4 to the AARE1 element of the *CHOP* promoter in the panel of thyroid cancer cells using ChIP assays. MG132 and bortezomib caused obvious recruitment of ATF4 to the AARE1 element of the *CHOP* promoter in FRO, KTC1, KTC2 and KTC3 cells, while binding of ATF4 was not significantly increased in 8305C and 8505C cells (Fig. 3D). It should be noted that the degree of ATF4 recruitment to the CHOP promoter (Fig. 3D) closely correlated with induction of CHOP in the panel of thyroid cancer cells (Fig. 3C), indicating that although ATF4 levels per se might not contribute to the differential induction of CHOP by proteasome inhibitors, the capability of ATF4 recruitment to the AARE1 element ascribes to the different induction of CHOP by proteasome inhibitors in the panel of thyroid cancer cells.

3.4. Implication of Nrf2 in the differential induction of CHOP by MG132 in the thyroid cancer cells

It was reported that Nrf2 and ATF4 were implicated in the regulation of CHOP on the contrary effects, ATF4 enhanced, while Nrf2 inhibited the expression of CHOP [18,25,30]. We then investigated the potential involvement of Nrf2 in the induction of CHOP by MG132 in thyroid cancer cells. Consistent with our previous report [34], Western blot analysis revealed various levels of Nrf2 expression in the panel of thyroid cancer cells, 8305C had the strongest expression, while FRO and KTC2 cells had the weakest expression under normal condition (Fig. 4A). Although MG132 caused an accumulation of Nrf2 in all the thyroid cancer cells, Nrf2 levels in MG132-treated FRO or KTC2 cells were much lower than those in vehicle-treated 8305C or 8505C cells (Fig. 4A). To confirm the contribution of Nrf2 to the induction of CHOP mediated by MG132, we used specific siRNA against Nrf2 (siNrf2) to suppress the accumulation of Nrf2 (Fig. 4B). siNrf2 significantly augmented CHOP induction mediated by MG132 in KTC1, KTC3, 8305C and 8505C cells, while it had no significant actions in FRO and KTC2 cells (Fig. 4C). To further confirm the implication of Nrf2 in MG132-mediated induction of CHOP, FRO and KTC2 cells, which demonstrated very low endogenous expression of Nrf2, were transfected with Nrf2 eukaryotic expression vector (Fig. 4D). Overexpression of Nrf2 significantly reduced CHOP induction mediated by MG132 (Fig. 4E).

3.5. Regulation of CHOP induction by Nrf2 via ATF4 recruitment to the CHOP promoter in the thyroid cancer cells

To clarify the possible mechanism(s) by which Nrf2 suppressed the induction of CHOP, we investigated the effects of Nrf2 on the recruitment of ATF4 to the AARE1 element on the *CHOP* promoter. Consistent with increased induction of CHOP (Fig. 4C), knockdown of Nrf2 by siNrf2 significantly increased the binding of ATF4 to the AARE1 element on the *CHOP* promoter in KTC1, KTC3, 8305C and 8505C cells, while had no obvious actions in FRO and KTC2 cells (Fig. 5A). In contrast, forced expression of Nrf2 reduced the recruitment of ATF4 to the AARE1 element on the *CHOP* promoter in FRO and KTC2 cells (Fig. 5B). Since it has been reported that Nrf2 interacts with ATF4 [37], binding between Nrf2 and ATF4 in Nrf2-transfected FRO cells was then examined. We found that ATF4 coimmunoprecipitated with Nrf2, and MG132 significantly increased their interaction (Fig. 5C). To demonstrate whether the interaction of Nrf2 and ATF4 affects recruitment of ATF4 to the *CHOP* promoter, re-ChIP was carried out. After the ATF4 first ChIP, no enrichment of Nrf2 binding was observed in the *CHOP* promoter (Fig. 5D), indicating that Nrf2 and ATF4 were not concurrently bound to the *CHOP* promoter. To test whether DNA binding is required for regulation of recruitment of ATF4 to the *CHOP* promoter by Nrf2, we used EGFP-tagged Nrf2-$^{\Delta}$NLS, Nrf2-$^{\Delta}$NES, and Nrf2-$^{\Delta}$NES/$^{\Delta}$TAD mutants which lack nuclear localization signal (NLS), nuclear export signal (NES) and nuclear export signal (NES)/transactivation domain (TAD), respectively [34]. FRO cells were transfected with these constructs and their expression were analyzed using fluorescent microscope (Fig. 5E). Consistent with a previous report [33], nuclear localization of Nrf2-$^{\Delta}$NLS was absent, while Nrf2-$^{\Delta}$NES and Nrf2-$^{\Delta}$NES/$^{\Delta}$TAD were predominantly localized in the nuclear (Fig. 5E). ChIP analysis demonstrated that Nrf2-$^{\Delta}$NLS had no effect on MG132-induced recruitment of ATF4 to the *CHOP* promoter, while wild-type (WT)-Nrf2, Nrf2-$^{\Delta}$NES and Nrf2-$^{\Delta}$NES/$^{\Delta}$TAD suppressed the enrichment of ATF4 to the CHOP promoter by MG132 with similar extent, indicating that nuclear translocation was sufficient, while DNA binding capacity of Nrf2 was not required for precluding recruitment of ATF4 to the *CHOP* promoter (Fig. 5F). In addition, another clinically used proteasome inhibitor bortezomib also caused recruitment of ATF4 to the CHOP promoter (Fig. 5G). The ATF4 recruitment induced by bortezomib was suppressed by wild-type (WT)-Nrf2, Nrf2-$^{\Delta}$NES and Nrf2-$^{\Delta}$NES/$^{\Delta}$TAD, while Nrf2-$^{\Delta}$NLS demonstrated no obvious effect on ATF4 recruitment (Fig. 5G). As PERK coordinates the convergence of ER stress with oxidative stress signaling via increasing ATF4 translation and promoting Nrf2 nuclear translocation [24], we used specific siRNA against PERK (siPERK) to investigate the potential involvement of PERK in CHOP induction mediated by proteasome inhibition. siPERK significantly decreased ATF4 accumulation (Fig. 5H) and CHOP induction (Fig. 5H and I) mediated by MG132 in FRO cells. However, siPERK demonstrated no obvious effects on EGFP-tagged Nrf2 nuclear translocation induced by MG132 in FRO cells (Fig. 5J).

3.6. Suppression of proteasome inhibition-mediated cytotoxicity of FRO cells by Nrf2, at least in part, via precluding CHOP induction

To study the function of precluding CHOP induction by Nrf2, we transfected FRO cells with Nrf2 and its mutants. Real-time RT-PCR demonstrated that WT-Nrf2, Nrf2-$^{\Delta}$NES and Nrf2-$^{\Delta}$NES/$^{\Delta}$TAD constructs significantly suppressed CHOP induction mediated by MG132 or bortezomib (Fig. 6A). In addition, these constructs demonstrated similar extent of suppressing effects on CHOP induction (Fig. 6A). However, Nrf2-$^{\Delta}$NLS mutant demonstrated no obvious effects on MG132 or bortezomib mediated induction of CHOP (Fig. 6A). MTT analyses demonstrated that WT-Nrf2, Nrf2-$^{\Delta}$NES and Nrf2-$^{\Delta}$NES/$^{\Delta}$TAD significantly suppressed MG132- or bortezomib-mediated cytotoxicity of FRO cells, while Nrf2-$^{\Delta}$NLS mutant demonstrated no obvious effects on cytotoxicity mediated by MG132 or bortezomib (Fig. 6B). Although these constructs suppressed CHOP induction with similar extents (Fig. 6A), compared with WT-Nrf2 and Nrf2-$^{\Delta}$NES, Nrf2-$^{\Delta}$NES/$^{\Delta}$TAD demonstrated lower suppressing effects on MG132- or bortezomib-mediated cytotoxicity of FRO cells (Fig. 6B). To further confirm the effects of CHOP on the suppressing effects of Nrf2, we cotransfected CHOP construct with Nrf2 and its mutants. CHOP overexpression almost completely blocked the suppressing effects of Nrf2-$^{\Delta}$NES/$^{\Delta}$TAD on cytotoxicity of FRO cells mediated by MG132 (Fig. 6C). The suppressing effects of WT-Nrf2 and Nrf2-$^{\Delta}$NES were also significantly blocked by CHOP overexpression (Fig. 6C).

4. Discussion

Proteasome inhibition affects different aspects of cell metabolism, blocks the clearance of damaged or misfolded proteins and the turnover of many key short-lived regulatory proteins, which is bound to expose cells to proteotoxicity. Recently, the apoptotic effects of proteasome inhibitors have been shown to be regulated at least in part by their ability to activate ER stress [3–7,36]. CHOP, also known as GADD153, a member of the bZIP family of transcription factors, has been shown to play an essential role in the response to a wide variety of cell stresses and induce cell cycle arrest and apoptosis in response to ER stress [38]. The basal expression level of CHOP is almost undetectable in most cell types, but its expression is rapidly induced by various stimuli at the transcriptional

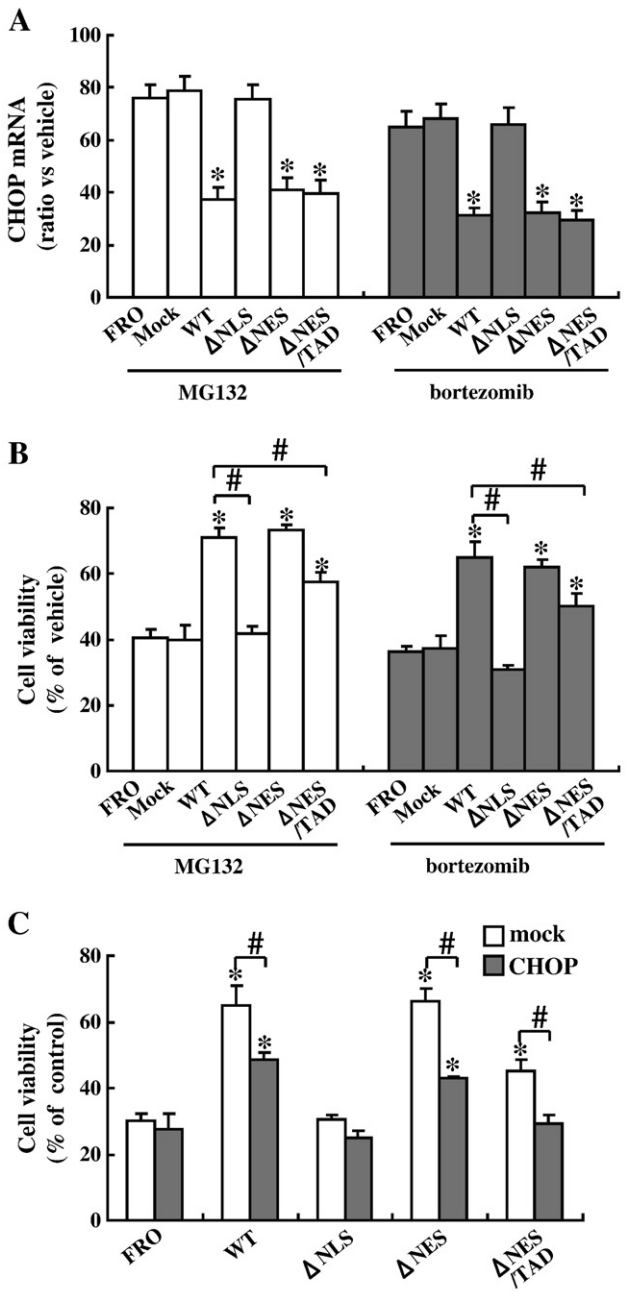

Fig. 6. Involvement of CHOP downregulation in the suppressing effects of Nrf2 on proteasome inhibition-mediated cytotoxicity of FRO cells. A, FRO cells were transfected with the indicated constructs for 24 h, then treated with 2 μM MG132 or 50 nM bortezomib for additional 8 h, and CHOP mRNA was analyzed using real-time RT-PCR. B, FRO cells were transfected with the indicated constructs for 24 h, then treated with 2 μM MG132 or 50 nM bortezomib for additional 24 h, and cell viability was measured using MTT assay. C, FRO cells were transfected with the indicated constructs for 24 h, then treated with 2 μM MG132 for additional 24 h, and cell viability was analyzed using MTT assay. *, $P<0.01$ *vs* control; #, $P<0.01$ *vs* the indicated.

and/or posttranscriptional level by a variety of agents that cause cellular stress [20]. We have previously shown that proteasome inhibitors increase CHOP expression at the transcriptional level and CHOP induction may contribute to the responsiveness of thyroid cancer cells to proteasome inhibition, with higher induction of CHOP in sensitive cell lines compared with less sensitive ones [7]. However, the mechanism by which proteasome inhibitors induced CHOP with different extents in thyroid cancer cells has not been well characterized.

CHOP have been reported to be induced through the activation of AARE, ERSE, and AP-1 elements of its promoter [15,19,39]. The elements controlling CHOP transcription vary depending on the cell type and the kind of stress induced [15–18,40]. In this study, we demonstrated that MG132 increased CHOP via activation of all these three elements of its promoter, but primarily by activation of the AARE1 element. We also found that the recruitment of ATF4 to AARE1 element of the *CHOP* promoter contributed to the differential induction of CHOP in a panel of thyroid cancer cells. In addition, we found that Nrf2 was also responsible for the differential CHOP induction by modulating the binding of ATF4 to the CHOP promoter. It is interesting to note that ATF4 and Nrf2 are strongly interconnected, as phosphorylation of Nrf2, which is necessary for its activation, can be carried out by PERK [26], which also contributes to the activation of the ATF4-CHOP pathway mediated by ER stress. While the role of ATF4 in the control of CHOP expression has been well established, little is known about the precise functions of Nrf2. In the current study, we observed accumulation of Nrf2 in thyroid cancer cells upon proteasome inhibition, which negatively regulated CHOP induction. Consistent with our data, it has been reported that CHOP expression correlates negatively with the presence of Nrf2; increased kinetics of CHOP mRNA accumulation in Nrf2-deficient fibroblasts following ER stress relative to that in wild-type fibroblasts, while Nrf2 overexpression attenuates CHOP accumulation during ER stress [26]. Possible mechanisms by which Nrf2 affects CHOP induction include the following: 1) Nrf2 acts as a direct transcriptional repressor at the CHOP promoter, 2) Nrf2 target genes directly or indirectly affect CHOP expression, 3) and Nrf2 precludes the binding of other transcription factors to the CHOP promoter. In the current study, we found that Nrf2 prohibited the recruitment of ATF4 to the CHOP promoter. Nrf2 binds DNA as a heterodimer, and ATF4 is one of its partners [37]. Coimmunoprecipitation confirmed that Nrf2 interacted with ATF4, and MG132 augmented their interaction. In addition, re-ChIP assay demonstrated that ATF4 and Nrf2 were not concurrently recruited to the *CHOP* promoter. Therefore, Nrf2 might preclude the binding of ATF4 to the CHOP promoter via their interaction. PERK is responsible for both ATF4 and Nrf2 activation, by which it coordinates the convergence of ER stress with oxidative stress signaling [24]. In the current study, we found that PERK was implicated in the ATF4 accumulation and CHOP induction mediated by MG132, while it had no obvious effects on nuclear translocation of Nrf2 in thyroid cancer cells. In addition to PERK, Nrf2 is reported to be phosphorylated by PKC, PI3K and MAPK, and phosphorylated modification by these kinases also promotes its nuclear translocation [24]. As a fact, we have previously reported that p38 MAPK is implicated in nuclear translocation and activation of Nrf2 upon proteasome inhibition [34].

Under normal physiological conditions, Nrf2 exists in its inactive form because of sequestration by the cytoplasmic nuclear translocation-inhibitor KEAP1, which delivers it to degradation by proteasome inhibition [41]. Release from KEAP1 and translocation to nucleus activates Nrf2 to initiate transcription of genes with antioxidant response element (ARE), most of which have roles in protecting the cell against oxidative and electrophilic stressors [27]. In the current study, we found that ROS generation was also implicated in the CHOP induction, as augmentation of ROS production by BSO and depletion of ROS generation by NAC increased and decreased the activation of the AARE1 element on the CHOP promoter, respectively. Considering the role of Nrf2 in the regulation of cellular defenses against ROS, modulation of ROS generation by Nrf2 target genes might be an alternative mechanism underlying the regulation of CHOP by Nrf2.

Collectively, this report provides a comprehensive analysis of the regulation of CHOP mRNA expression in a panel of thyroid cancer cells which have different responsiveness to proteasome inhibitors.

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