Identification of the Functional Autophagy-Regulatory Domain in *HCLS1*-Associated Protein X-1 That Resists Against Oxidative Stress

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HCLS1 Associated Protein X-1 (HAX1) promotes cell survival through attenuation of the damaged signals from endoplasmic reticulum and mitochondria, which are known as prominent intracellular compartments for the autophagic process under stress conditions. This study investigates whether autophagy can be upregulated in response to HAX1 overexpression and identifies the functional motif in HAX1 responsible for the autophagic induction. Autophagosome accumulation, mitochondrial membrane potential ($\Delta\psi_m$), and apoptosis were assessed in HEK293 cells post transduction with full-length or truncated HAX1-encoding genes, while empty vector-transduced cells served as control. Upon the oxidative stress, the enhanced autophagy induction was observed in cells overexpressing HAX1, as well as HAX1 truncations that encode peptide segments ranging from amino acids 127–180 (AA127-180). This protective response was further supported by flow cytometry and Western Blot results, in which oxidative stress-induced $\Delta\psi_m$ dissipation and the programmed cell death were suppressed in HAX1-overexpressing cells, associated with reduced DNA fragmentation and decreased Caspase-9 cleavage. Interestingly, the HAX1-induced autophagy response was abrogated when AA127-180 was removed, compromising the antiapoptotic effects upon oxidative stress. Overall, these data indicate that autophagy induction is involved in HAX1-induced cell protective mechanism, and AA127-180 serves as the functional autophagy-regulatory domain of this antiapoptotic protein.

Keywords: HAX1, autophagy, functional domain, mitochondria, apoptosis

Introduction

HCLS1 ASSOCIATED PROTEIN X-1 (HAX1) is a multiple functional regulator involved in mediating cell proliferation, apoptosis, granulopoiesis, mobility, and contractility (Zhao *et al.*, 2009; Skokowa *et al.*, 2012; Liu *et al.*, 2015; Wang *et al.*, 2015). Particularly, the HAX1-mediated cell survival response plays a critical role in the molecular mechanism for tumor biogenesis by enhancing lymphomagenesis in HAX1 signal-disrupted B cells, which is associated with an attenuated apoptotic response (Baumann *et al.*, 2014). Recent studies have also revealed a reduction in HAX1 levels in ischemic hearts. Overexpression of this antiapoptotic protein can promote cardiomyocyte survival through attenuation of detrimental responses in endoplasmic reticulum (ER) and mitochondria (Lam *et al.*, 2013, 2015), both of which are prominent cellular machineries involved in the regulation of autophagy response.

The ER is the primary intracellular compartment responsible for protein synthesis, modification, and trafficking, while mitochondria are the prominent double membrane-bound system in the maintenance of cellular energy supply through producing adenosine triphosphate. Under stress condition, the fidelity of protein translation and efficiency of oxidative phosphorylation are compromised, respectively, in ER and mitochondria. Autophagy plays a central role in the regulations of ER turnover and mitochondrial reconstitution in response to this stress, which are demanded to meet different cellular metabolic requirements. Indeed, FAM134 reticulon protein family has been recently identified as ER-resident receptors to facilitate autophagy-induced ER degradation (ER-phagy) through physical interaction with several autophagy components, including LC3 and GABARAP (GABAA receptor associated protein). Correspondingly, such a selective ER-phagy was abrogated in *FAM134*-knockout mouse, leading to the ER membrane

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expansion, ER turnover inhibition, and apoptotic cell death (Khaminets *et al.*, 2015). In the other aspect, upon receiving injury signals, the damaged mitochondria can be recognized and selectively removed through autophagy-mediated degradation (also called mitophagy), which protects against further cellular dysfunction and eventually programmed cell death. Such an intrinsic protective mechanism is initiated through accumulation or posttranslational modification of several protein- or lipid sensors, such as PINK1/Parkin and C18-ceramide, at the outer mitochondrial membrane, and then mitochondrial sequestration is performed through binding LC3 to mitophagy receptors, consequently forming autophagosomes through a LIR (LC3-interacting region) motif.

The involvement of autophagy in the pathological development of various diseases has been widely accepted. Upregulation of autophagy has been observed in several types of cancer and can promote cancer cell survival upon microenvironmental stress to increase growth and metastasis (Hu *et al.*, 2017; Katheder *et al.*, 2017). Conversely, failed autophagy induction compromises the survival capacity of cardiomyocytes and neurons under stress conditions, leading to heart failure and nerve damage. Indeed, impairment of autophagic flux will result in the accumulation of dysfunctional mitochondria, the release of damaged reactive oxygen species, and consequently the initiation of cell death program. Intriguingly, targeting at autophagy has provided a novel chance for disease treatment. A most recent study revealed that D-Tat-Beclin-1, a cell-permeable Atg6/Beclin1-derived autophagy-inducing peptide, could reduce cardiac triglyceride by lipoprotein lipase, which will consequently improve heart performance in diabetic mouse (Hua *et al.*, 2015). Another study indicated that blockade of the interaction between TRB3 and p62, using a synthetic peptide, can inhibit metastasis and promote antitumor efficacy through impeding autophagic-proteasomal degradation in cancer cells (Hua *et al.*, 2015).

Previous studies have revealed the involvement of HAX1 in the anti-death machinery through attenuating ER stress and reducing the opening of the mitochondrial permeability transition pore. Therefore, it would be interesting to investigate whether autophagy also contributes to the HAX1-induced cell protective mechanism (Lam *et al.*, 2013, 2015). In this study, the autophagy response, along with mitochondrial function and apoptotic signals, is investigated in HAX1-overexpressing cells subjected to oxidative stress. Such a context of biological responses was also assessed in cells overexpressing HAX1 truncations, to identify the effective functional motif responsible for autophagy induction.

Materials and Methods

Construction of full length and truncations of HAX1

Total RNA was extracted and purified from human heart tissue, which is described as previous study (Cai *et al.*, 2015a). Full-length or truncated HAX1 encoded cDNA was generated and amplified using primers (Supplementary Tables S1 and S2; Supplementary Data are available online at www.liebertpub.com/dna), which were designed according to the nucleotide sequence of *Homo sapiens* HAX1 mRNA (NM_006118.3). These cDNA segments were then constructed into the corresponding restriction enzyme sites

of the eukaryotic expression vectors pEGFP-C1 (Cat. No.: 6084-1; Addgene) and p3xFLAG-CMV-7.1 (Cat. No.: E4026; Addgene), allowing for expression of these exogenous fusion proteins to be indicated or detected by green fluorescent protein (GFP) or FLAG signals. These recombinant plasmids were then transfected into HEK293TN cells using Lipofectamine[®] 2000 Reagent (Cat. No.: 11668-30; Thermo Fisher Scientific). After 5 h, the transfection reagent was removed, and cells were maintained in 10% fetal bovine serum (FBS)-supplemented Dulbecco's Modified Eagle Medium (DMEM) (Cat. No.: D6546; Sigma-Aldrich). Cells were available for further experiments after 48 h in culture.

Immunofluorescence staining

Cells were grown on coverslips and fixed with 4% buffered paraformaldehyde post solvent vehicle or H₂O₂ treatment. After a double rinse with phosphate-buffered saline (PBS), cells were permeabilized with 0.1% saponin PBS. Slides were blocked with goat serum (Cat. No.: 005-000-121; Jackson ImmunoResearch, Inc.) and incubated with LC3 antibody (Cat. No.: 12741; Cell Signaling Technology) at 4°C overnight. Cells were then washed with PBS thrice and were stained with goat anti-rabbit Alexa 594 (1:200) (Cat. No.: A-11037; Thermo Scientific) for 30 min at 37°C and then washed thrice with PBS and mounted on glass slides. Nuclei were visualized with 4, 6-diamidino-2-phenylindole staining. Images were acquired using a confocal microscope (Zeiss LSM 510).

Assessment of mitochondrial membrane potential

As previously described (Cai *et al.*, 2015b), cells were harvested at 0, 1, 2, 3, and 4 h after exposure to urea hydrogen peroxide (1 mM) loaded with 10 nM TMRE (tetramethylrhodamine, ethyl ester) dye (Cat. No.: T669; Invitrogen) at 37°C for 30 min. After exposure to hydrogen dioxide, the loaded cells were excited at 568 nm, and the magnitude of emitted fluorescence was measured at 630 nm. TMRE intensity was recorded using flow cytometry according to the cell number distribution. Finally, FlowJo software (Tree Star, Inc.) was used to calculate the mean integral fluorescence density to indicate $\Delta\psi_m$.

DNA fragmentation

Levels of cytosolic mono- and oligonucleosomes were examined using an ELISA assay according to the manufacturer's instructions (Cat. No.: 11585045001; Roche Applied Science). Briefly, HEK293TN cells were labeled with Bromodeoxyuridine and then seeded into a six-well plate. After incubation with urea hydrogen peroxide, cells were lysed and centrifuged at 13,000 rpm for 10 min to remove the nuclei. The supernatant was subsequently incubated in an anti-DNA antibody-coated 96-well plate overnight. On the second day, ABTS solution (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) was applied for color development after incubation with immune reagent for 2 h and, subsequently, washed out. The extent of DNA fragmentation was assessed with a plate reader at 405 nm.

Cell cycle analysis

Cells were trypsinized and harvested at 0, 5, and 12 h post serum starvation-induced synchronization. After cells were washed twice with cold PBS and fixed with 70% ethanol at

4°C, they were subjected to RNase/propidium iodide (PI) staining using a Cell Cycle Kit (Cat. No.: F10797; FxCycle™ PI/RNase staining solution, Thermo Scientific) according to the manufacturer's instructions. Cells were analyzed using flow cytometry (BD FACS Canto I), and a minimum of 10,000 events was analyzed at 561 nm excitation wavelength. Data were analyzed using computational software FlowJo (Version 10, Tree Star, Inc. Ashland, OR). The distribution of cell populations in phases of G0/G1, S, and G2/M was calculated using the Dean-Jett-Fox mathematical model (Lai *et al.*, 2012).

Transfection of cells with tandem fluorescent LC3 plasmid

A monolayer of HEK293 cells, in which FLAG-tagged HAX1 or HAX1 truncations have been overexpressed, was transfected 48 h before the experiment with ptfLC3, (Plasmid #21074, Addgene) (Kimura *et al.*, 2007) with 1 µg per well of a six-well plate using the Lipofectamine 2000 Reagent. Green and red fluorescence were visualized and captured in cells under basal conditions or after exposure to H₂O₂ stimulation using a confocal microscope (Zeiss LSM 510). Yellow dots that co-localized green and red fluorescent signals were then used to indicate autophagosomes, while the aggregated red fluorescent dots were used to indicate autolysosomes.

Assessment of LC3 expression by flow cytometry

After 1 h of exposure to H₂O₂ (0.5 mM) or vehicle solvent, cells were trypsinized and washed with PBS thrice. Cells were exposed to permeabilization solution (0.1% saponin in PBS) and then incubated with phycoerythrin (PE)-conjugated LC3 antibody (Cat. No.: 13611; Cell Signaling Technology) at 37°C for 1 h. After washing with PBS (containing 5% FBS), fluorescence signals from PE excited at 565 nm were collected at an emission wavelength of 575 nm in >10,000 cells per sample. FlowJo software (Tree Star, Inc.) was used to generate a cell distribution diagram according to PE fluorescence intensity, and the median value of PE fluorescent intensity was then calculated to indicate the expression of LC3.

Assessment of MTDR fluorescent intensity by flow cytometry

As previously described (Mauro-Lizcano *et al.*, 2015), after exposure to H₂O₂ (0.1 mM for 12 h) alone or combined treatment of H₂O₂ and hydroxychloroquine (lysosomal inhibitor), cells were trypsinized for 5 min at 37°C and resuspended in complete medium with 10 nM MitoTracker Deep Red (MTDR) (M22426; Invitrogen) and then incubated for 15 min at 37°C. After washing with PBS (containing 5% FBS), fluorescence signals from MTDR excited at 644 nm were collected at an emission wavelength of 665 nm in >10,000 cells per sample. FlowJo software (Tree Star, Inc.) was used to generate a cell distribution diagram according to MTDR fluorescence intensity, and the median value of MTDR fluorescent intensity was then calculated.

Assessment of apoptosis using flow cytometry

Cells were gently washed and stained with PE-Cy7-conjugated Annexin V (eBioscience) for 25 min. Stained cells were then washed gently and resuspended in solution containing

eFluor-780 (eBioscience). Fluorescence signals from PE-Cy7 and eFluor 780 were excited at 488 and 633 nm and were collected at emission wavelengths of 767 and 780 nm, respectively, in >10,000 cells per sample group. FlowJo software (Tree Star, Inc.) was used to generate a cell distribution diagram according to fluorescence intensity and to calculate the percent of Annexin V positive apoptotic cells in the total population.

Western blot

Western blot analysis was performed as previously described (Cai *et al.*, 2015a). Membranes were blocked with 5% milk and then probed with specific antibodies against GFP Tag (Cat. No.: MA5-15256; Thermo Fisher Scientific), FLAG Tag (Cat. No.: MAB8529; R&D Systems), HAX1 (Cat. No.: 610824; BD Biosciences), Bax (Cat. No.: 2772; Cell Signaling Technology), Bcl-2 (Cat. No.: 2876S; Cell Signaling Technology), Bcl-xL (Cat. No.: 2762S; Cell Signaling Technology), Caspase-9 (Cat. No.: 9502T; Cell Signaling Technology), Cleaved Caspase-9 (Cat. No.: 9501T; Cell Signaling Technology), Beclin1 (Cat. No.: NB500-249; Novus Biologicals), LC3 (Cat. No.: NB100-2220; Novus Biologicals), and GAPDH (Cat. No.: G9545; Sigma Aldrich). Horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies (1:5000) were obtained from GE Healthcare Life Sciences. Membranes were developed using an enhanced chemiluminescence Western blot analysis detection system (Amersham Biosciences). All protein levels were quantified using AlphaEase FC software (Alpha Innotech, San Leandro, CA).

Statistical analysis

Data are expressed as mean ± SEM. At least six independent experiments were used in each assessment, and each experiment was considered as a single *n*. Tests for normality and homoscedasticity were performed to justify the normality of the distribution and the equality of variance. Comparison of the results among groups was then analyzed using ANOVA, and Bonferroni exact test was conducted for *post hoc* analyses (SPSS 13.0, IBM Co., Armonk, NY). Probability (P) values of <0.05 were considered to be significant.

Results

Overexpression of full-length or truncated HAX1 encoding genes in HEK293 cells

As shown in Figure 1A, full-length (g) and truncated HAX1 encoding genes were constructed into eukaryotic expression vectors. The GFP expressing gene was tagged and fused at the N-terminus of each recombinant protein, while the empty plasmid vector (m) was treated as control. Truncated proteins (a-f) were expressed from the N-terminus containing Bcl-2 homology (BH) domains, but not the C-terminus of HAX1. The expressions of truncated proteins (h, i) ended at the C-terminus of HAX1 without containing N-terminal protein sequences. Western blot analysis was used to assess proper function of all vectors 48 h post liposome-mediated transfection, which was detected using GFP antibody and evidenced by the appearance of GFP signals on the bands corresponding to their respective molecular weight (Fig. 1B). Both flow cytometry (Fig. 1C) and immunofluorescent microscopy (Fig. 1D) showed green fluorescent signal in a large percentage of cells

FIG. 1. Exogenous expression of HAX1 truncates in HEK293 cells. **(A)** Schematic diagram of HAX1 full-length cDNA and various truncates, in which GFP epitope tag was fused at the N-terminal of HAX1 or HAX1 truncations' cDNA. **(B)** Representative western blot indicated the expression of various HAX1 truncates with GAPDH used as internal control. **(C)** Flow cytometry analysis showed the percentage of GFP positive cell population after transduction with HAX1 truncate-encoding vectors. **(D)** Appearance of *green* fluorescence indicated successful transduction of HAX1-encoding vectors into HEK293 cells. HAX1, *HCLS1* associated protein X-1; GFP, green fluorescent protein; MW, molecular weight.

(~85%), and there were no morphological changes detected in these cells post gene transfection.

Although the pro-proliferative effect of HAX1 has been revealed in several studies (Li *et al.*, 2015; Wang *et al.*, 2015), it remains unclear whether this function is retained in response to the removal of the functional domains from HAX1. Thus, the cell cycle (especially the DNA synthetic (S) phase) was analyzed post transfection with full-length or truncated HAX1. Cells were synchronized using serum deprivation, which was evidenced by the similar cell population that distributed in the S phase (~40%). The population in S phase was increased to 54% and 58% in empty-vector transfected cells at 5 and 9 h, respectively, after exposure to serum (Supplementary Fig. S1A, B). In the presence of full-length HAX1, more than 67% cells were distributed in the S phase after 5 h of serum exposure. This pronounced that pro-proliferative response was also revealed in the HAX1 truncations-d, e, f, h, i, j, and k, but was not observed in truncations a, b, c, and l (Supplementary Fig. 1A, B). Further analysis showed that pro-proliferative effect only appeared in the truncations containing HAX1 amino acid sequences 127–180 (Supplementary Fig. S1C), suggesting the importance of motif in promoting cell proliferation.

Autophagic response in the presence of oxidative stress

Autophagy is a protective and programmed cellular biological process that has evolved into an intrinsic quality control mechanism to keep cells from damage caused by excess or detrimental macromolecules. Microtubule-associated protein 1A/1B-light chain 3 (LC3) is a soluble protein and a major component involved in the formation of

autophagosomes. Under basal conditions there was no difference in LC3 expression among cells overexpressing full-length HAX1 or HAX1 truncations (Fig. 2A). Flow cytometry analysis showed that the expression level of LC3 was enhanced to a greater level in HAX1-overexpressing cells (g) compared with control group (m) (Fig. 2A, B) upon onset of H₂O₂-induced oxidative stress. A significant upregulation of LC3 was also observed in HAX1 truncations d, e, f, h, i, j, k with the appearance of autophagosome signal in both cells overexpressing full-length HAX1 and these truncations (Fig. 2A–C). In the early stages of autophagy, the cytosolic form of LC3 (LC3-I) can be converted to phosphatidylethanolamine-conjugated form (LC3-II), which subsequently initiates the formation and lengthening of the autophagosome. Thus, the western blot signal ratio between LC3-II and LC3-I can be used to determine the alterations in the extent of autophagy. As shown in Figure 2D, the ratio of LC3-II to LC3-I has correspondingly increased in above cells overexpressing full-length HAX1 (g) and truncations (d, e, f, h, i, j, k), which significantly outnumbered that of the control group (m). Such pronounced autophagic responses were not observed in the other HAX1 truncations (a, b, c, l) (Fig. 2A, C), and the LC3-II/LC3-I ratios in HAX1 truncations (a, b) were below those of the control group (m) ($p < 0.05$) (Fig. 2D). It is noteworthy that the strengthened autophagic induction appeared only in cells with the over-expressed truncations that contained amino acids 127–180 of HAX1, suggesting that the functional autophagy regulatory domain of HAX1 is located within this motif.

Autophagic flux is a measure of autophagic degradation activity (Klionsky *et al.*, 2012). To determine the effective

FIG. 2. Autophagy formation in HAX1-overexpressing cell response to oxidative stress. HEK293 cells were overexpressed with GFP-tagged HAX1 or truncations and then exposed to 0.5 mM H_2O_2 for 1 h. (A) Representative flow cytometric histogram illustrating the distribution of LC3-expressing cells at the basal or oxidative stress condition after exposure to H_2O_2 . (B) Quantitative analysis of LC3 expression level in H_2O_2 -exposed HEK293 cells using MFI (median fluorescence intensity). (C) Confocal immunofluorescent microscopy revealed the appearance of LC3-autophagosome in cell response to oxidative stress. (D) Representative western blots illustrate the expression of LC3, Beclin-1, and GAPDH. Autophagic flux was assessed by comparing LC3-II/LC3-I ratio ($n=6$ cell preparations, $*p<0.05$). MW, molecular weight.

HAX1 fragment responsible for the regulation of this degradation activity, HAX1 full-length cDNA and several truncations were tagged with FLAG epitope and then expressed in HEK293 cells (Supplementary Fig. S2A, B). An enhanced ratio of LC3-II to LC3-I was observed in cells with the FLAG-tagged truncations that contain amino acids 127–180 of HAX1 (Supplementary Fig. S2C, D) upon H_2O_2 -induced oxidative stress. These cells were subsequently transfected with a ptf-LC3 vector, enabling quantitative assessment of autophagic flux by monitoring the appearance of autophagosome (yellow dots) and autolysosomes (red dots) in cells prior- and postoxidative stress (Fig. 3 and Supplementary Fig. S3). As is shown in Supplementary Figure S3A, both GFP-LC3 and RFP-LC3 distributed loosely in the cytoplasm under basal conditions. Upon 15 min oxidative stress stimulation, green fluorescent signal aggregated and co-localized

with red fluorescent signals, indicating the formation of an autophagosome (Supplementary Fig. S3B). It is noteworthy that the enhanced amount of autophagosome was observed in cells with the HAX1 truncations containing amino acids 127–180 (Fig. 3A, B). Interestingly, the autophagosome in these cells was removed by lysosome at the 30 min postoxidative stress (Supplementary Fig. S3C), as evidenced by the increased number of red fluorescent dots (Fig. 3A, C). These results indicated that autophagic flux is tuned up in HAX1 truncations that contain amino acid 127–180.

Dissipation of mitochondrial membrane potential upon oxidative stress

Mitochondrial integrity and energy metabolism rely on autophagy-mediated degradation of damaged macromolecules, and proper autophagy is dependent on healthy mitochondria (Okamoto, 2011). Mitochondrial function was therefore examined by assessing membrane potential ($\Delta\psi_m$) in the presence of oxidative stress (Fig. 4A), and $\Delta\psi_m$ in cells expressing HAX1 or truncations (blue curve) were compared with cells expressing empty plasmid (pink curve) at several time points (Fig. 4B). Oxidative stress resulted in the dissipation of $\Delta\psi_m$ in cells with empty vector, and $\Delta\psi_m$ was reduced by 62% at 4 h postexposure to H_2O_2 . Overexpression of HAX1 significantly impeded this decay process while maintaining mitochondrial function at more than 70% compared to basal conditions (Fig. 4B-g). Interestingly, this protective effect against mitochondrial damage was revealed in most HAX1 truncations (Fig. 4B-d, e, f and Fig. 4B-h, i, j, k, l) and contained the C-terminal sequence of HAX1, indicating the significance of this protein domain. Mitophagic flux is essential for maintaining mitochondrial integrity and function (Koentjoro *et al.*, 2017), and such a selective autophagic elimination of damaged mitochondria can be quantitatively determined by a flow cytometry-based approach using MTDR (Mauro-Lizcano *et al.*, 2015). As is shown in Figure 5, the pronounced mitophagic flux was observed in cells that overexpress HAX1 truncations d, e, f, g, h, i, j, k and l post-oxidative stress, suggesting that HAX1 C-terminal-induced mitochondrial protection is partially contributed by the up-regulated autophagic flux response.

Identification of HAX1 functional domain that resists oxidative stress-induced apoptosis

Programmed cell death is initiated in response to sustained oxidative stress. Indeed, the presence of phosphatidylserine on the external leaflet of the plasma membrane (an early apoptotic event that can be detected using Annexin V staining) was observed in HEK293 cells postexposure to H_2O_2 at several concentrations (Supplementary Fig. S4A). During the late stage of apoptosis, loss of plasma membrane integrity allows cellular uptake of viability dyes such as eFluor 780. Cells in the present study were co-stained with PE-Cy7-conjugated Annexin V and eFluor 780 after exposure to 1 mM H_2O_2 for 12 h. The events of early-stage apoptosis, late-stage apoptosis, necrosis, and survival were indicated by pseudo-color dots in quadrants Q1–Q4, respectively, as indicated by flow cytometry analysis (Fig. 6A). Although there were no significant differences in early-stage apoptotic and necrotic events among all groups in the presence of H_2O_2 ,

FIG. 3. Autophagic flux in HAX1-overexpressing cell response to oxidative stress. The ptf-LC3 vector was transfected into HEK293 cells that overexpress Flag-tagged HAX1 or truncations. **(A)** Green and red fluorescence and their co-localized signals were monitored in cells under basal condition or after exposure to H₂O₂ (0.5 mM) at 15 and 30 min. **(B)** Quantitative analysis of autophagosomes by counting the yellow dots (co-localized signals of green and red fluorescence) appeared in the cytoplasm. **(C)** Quantitative analysis of autolysosomes by counting the red fluorescent aggregates in the cytoplasm ($n=6$ cell preparations, $*p<0.05$).

FIG. 4. Oxidative stress induced the dissipation of mitochondrial membrane potential. After transduction with GFP-tagged full-length or truncated HAX1, cells were loaded with TMRE and then exposed to H₂O₂ (1 mM). **(A)** Mitochondrial membrane potential ($\Delta\psi_m$) was assessed using flow cytometry at basal condition at 1, 2, 3, and 4 h postexposure to H₂O₂. **(B)** Quantitative analysis of the mean intensity of TMRE illustrates temporal $\Delta\psi_m$ alterations of H₂O₂-treated cells in the presence of either full-length or truncated HAX1 ($n=7$ cell preparations, $*p<0.05$). TMRE, tetramethylrhodamine, ethyl ester.

FIG. 5. Mitophagic flux in HAX1-overexpressing cells upon oxidative stress. HEK293 cells overexpressing Flag-tagged HAX1 or truncations were loaded with MTDR. The MTDR fluorescence intensity in HEK293 cells was assessed by flow cytometry under basal condition and in the presence of H_2O_2 (0.1 mM for 12 h) and lysosomal inhibitor (hydroxychloroquine). Mitophagic flux was determined by the ratio of MTDR fluorescence in the presence of hydroxychloroquine, normalized to the corresponding value in nonhydroxychloroquine-treated cells ($n=6$ cell preparations, $*p<0.05$). MTDR, MitoTracker Deep Red.

H_2O_2 -induced late-stage apoptotic events were significantly suppressed in HAX1-overexpressing cells. This inhibitory effect was also observed in cells overexpressing HAX1 truncations with amino acid sequences 127–180 (d, e, f, h, i, j, k), but not in truncations (a, b, c, l) (Fig. 6A, B). Correspondingly,

antiapoptotic effects in HAX1 and these truncations were further supported by the reduction of internucleosomal DNA fragmentation, a hallmark of the nuclear event (Fig. 6C).

Inhibition of the prominent regulators in the mitochondrial apoptotic pathway

Bax is a nuclear-encoded cytosolic protein that can pierce mitochondrial outer membranes to mediate apoptosis, while Bcl-2 serves as an intrinsic antagonist that can block this damage process. Western blot assessment indicated that H_2O_2 -induced upregulation of Bax was associated with a reduction of Bcl-2 in a dose-dependent manner (Supplementary Fig. S4B). As shown in Figure 6A and B, a decreased ratio of Bax over Bcl-2 was observed in HAX1-overexpressing cells under oxidative stress conditions, which was significantly less compared with control group (m). Such a decrease in Bax/Bcl-2 ratio was also revealed in cells with truncations (d, e, f, h, i, j, k) (Fig. 7A, B), which supports the evidence above showing a significant protective effect on mitochondrial function (Fig. 4). Following permeabilization of the mitochondrial outer membrane, cytochrome c is released to function in the activation of caspase-9 through a proteolytic process. In agreement with a previous study (Han *et al.*, 2006), oxidative stress-induced caspase-9 activation was inhibited in response to HAX1 overexpression (g), as evidenced by the reduced cleavage level at amino-acid residue Aspartate315, compared with the control group (m) (Fig. 7A, B). Interestingly, the similar caspase-9 inhibitory effect also developed in HAX1 truncations containing the protein motif 127–180.

FIG. 6. The oxidative stress-induced apoptotic responses in HAX1-overexpressing cells. (A) HEK293 cells were overexpressed with GFP-tagged HAX1 or truncations. Representative flow cytometric pseudo-color density plots illustrating the distribution of PE-Cy7 positive and eFluo-780 positive events under the basal condition and postexposure to H_2O_2 (1 mM) for 12 h. Quadrants indicate the appearance of early apoptotic (Q1), late apoptotic (Q2), necrotic (Q3), and live cells (Q4), respectively. (B) Quantitative analysis of late apoptotic events in the absence or presence of H_2O_2 . (C) DNA fragmentation (mono- and oligonucleosomes) in HEK293 cells overexpressing with either full-length or truncated HAX1 was detected by ELISA in the presence and absence of H/R injury ($n=6$ cell preparations, $*p<0.05$).

FIG. 7. The changes in the protein levels of apoptosis regulatory molecules in the presence of oxidative stress. HEK293 cells were exposed to H_2O_2 (0.8 mM) for 12 h, and cell lysates were used to perform immunoblot analysis. **(A)** Representative Western blots illustrating expression levels of cleaved Caspase-9, Caspase-9, BAX, Bcl-2, and GAPDH. **(B)** Quantitative analysis of the ratios of BAX to Bcl-2 obtained from the immunoblots. **(C)** Activation of Caspase-9 was indicated using the ratio of cleaved Caspase-9 to Caspase-9 obtained from the immunoblots ($n=6$ cell preparations, $*p < 0.05$). MW, molecular weight.

Discussion

Several mechanisms contribute to the anti-cell death effects of HAX1, including attenuation of ER stress (Lam *et al.*, 2013; Abdelwahid *et al.*, 2016), blockade of Caspase activation (Han *et al.*, 2006; Yan *et al.*, 2015), and protection against the disruption of mitochondrial membrane integrity (Lam *et al.*, 2015). This study provides the first evidence that HAX1-induced antiapoptosis is associated with an upregulated autophagic response. Importantly, we identified a region between amino acids 127 and 180 of HAX1 that serves as a functional domain in autophagy induction. Upon the oxidative stress, overexpression of this domain can tune up the autophagic response to the same level as that of full-length HAX1, associated with the inhibition of mitochondrial apoptotic signaling pathway and consequently reducing apoptosis.

An initial study purported HAX1 as a Bcl-2 family protein on the basis of sequence similarities with other Bcl-2 family members, including the appearance of BH1 and BH2-like motifs at position 37–56 and 74–89 in HAX1, respectively

(Sharp *et al.*, 2002). Such conservative domains commonly appear in antiapoptotic Bcl-2 family proteins (including Bcl-2, Bcl-xL, and Bcl-xw), which serve as molecular signature of endogenous inhibitors against multidomain pro-apoptotic proteins. However, analysis of the secondary structures revealed that HAX1 is not considered to be a member of the Bcl-2 family-related protein due to the lack of stable hairpins within BH1 and BH2 modules, which are formed by hydrophobic α -helices (Jeyaraju *et al.*, 2009). Our present study provided evidence that HAX1 can upregulate the autophagic response and convey an antiapoptotic effect regardless of the occurrence of these BH domains at the N-terminus. It is noteworthy that a Proline-Glutamic acid-Serine-Threonine (PEST) sequence-enriched region is located at the amino acid residues 104–117 of HAX1, and this motif functions as a proteolytic signal to convey ubiquitin-induced proteasomal degradation (Li *et al.*, 2012a). Indeed, pharmacology protein synthesis inhibitor-induced apoptosis was decreased in cells overexpressing PEST sequence-deleted HAX1, associated with a reduction in degradation of HAX1 (Li *et al.*, 2012a). Although proteasomal degradation was not investigated in the

current study, PEST sequence deficiency did not enhance antiapoptotic effect to a greater level compared to that of full-length HAX1, indicating that ablation of this intrinsic degradation signal alone might be insufficient to tune up the anti-death molecular mechanism under conditions of oxidative stress.

Amino acids 117–279 of HAX1 contain multiple protein–interaction motifs that are involved in the regulation of inflammatory responses, cell migration, calcium handling, and mRNA surveillance (Fadeel and Grzybowska, 2009). Particularly, two antiapoptotic domains (located at amino acid motifs 187–235 and 175–206) have been identified as the binding sites that interact with HtrA2/Parl and caspase-9, respectively, both of which are critical signaling modules in the mitochondrial apoptosis pathway. Induction of autophagy can remove damaged mitochondria after oxidative stress, serving an essential role in maintaining mitochondrial function and integrity for cell physiology and survival (Tong and Sadoshima, 2016). A few studies have reported the regulation of HAX1 on autophagy in the stress condition (Appaswamy *et al.*, 2009; Li *et al.*, 2010). The enhanced expression of Beclin-1, an autophagy essential protein, was observed in HAX1-deficient neutrophils, and overexpression of HAX1 inhibited the formation of autophagosomes upon the starvation, suggesting that HAX1 serves as negative regulator of stress-induced autophagy response. However, our current study revealed that HAX1 overexpression can promote the autophagy response, associated with the pronounced antiapoptotic effect, in the presence of H₂O₂-induced oxidative stress. This discrepancy with previous reports may be due to the different experimental protocols that were used to simulate stress condition. Interestingly, our study indicated that HAX1-induced upregulation of autophagy was a function of the occurrence of the amino acid motif 127–180, and ablation of this domain compromised autophagic and antiapoptotic responses associated with reduced mitochondrial protection in the presence of oxidative stress. In agreement with previous studies, caspase-9 inhibition was observed in cells overexpressing HAX1 truncates that contained the caspase-9 binding domain (amino acid sequence 175–206) (Han *et al.*, 2006). Interestingly, this inhibitory effect was not revealed in cells that were transduced with HAX1 truncate (180–279), suggesting that the amino acid sequence 175–180 is a critical factor in mediating caspase-9 activation.

While enhanced expression levels of HAX1 are associated with an increased proliferative capacity of human tumor cells (Wang *et al.*, 2015; Yan *et al.*, 2015), HAX1 gene silencing significantly decreased cell proliferation both *in vitro* and *in vivo* (Li *et al.*, 2015). These data represent the first evidence that DNA replication is promoted in HAX1-overexpressing cells. Indeed, the activities of several cell cycle regulators (including p53 and Grb7 [growth factor receptor bound protein 7]) can be mediated by HAX1 (Banerjee *et al.*, 2009; Qian *et al.*, 2016). Recent studies using a yeast two-hybrid system have demonstrated that HAX1 can bind to Salvador homolog 1 (SAV1) (Luo *et al.*, 2011; Li *et al.*, 2012b), and such an interaction is potentially involved in the regulation of proliferation through mediation of the Hippo–Yap signaling cascade. Inactivation of SAV1 can tune up Hippo signaling and promote the nuclear entry of Yap, which is required for S-phase entry by regulating the transcription of genes involved in the assembly and/or firing of replication

origins and homologous recombination of DNA (Shen and Stanger, 2015). The most recent study also revealed a non-transcriptional function of Yap that is involved in cytokinesis, which is demanded for the stability of both midbody and spindle during mitosis (Bui *et al.*, 2016). Importantly, the amino acid sequence 127–180 in HAX1 was identified as the functional domain in the regulation of cell cycle. Such a finding provides an opportunity to explore the signaling pathways that contribute to HAX1-induced proliferation.

In summary, the protein motif 127–180 appears to serve as a crucial functional domain responsible for HAX1-induced cell cycle progression, autophagy induction, and mitochondrial protection. Particularly, occurrence of this motif is important in maintaining cell survival under oxidative stress conditions, giving it potential to be used as a therapeutic strategy for cancer treatment. Blockage of the domain can inhibit the biological function of HAX1, which may protect against tumor growth through inhibition of cell proliferation and survival. Finally, the polypeptide analogy can also be generated and developed according to the protein conformation of this functional domain to induce tissue repair and promote regeneration.

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