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Glyceraldehyde-3-phosphate dehydrogenase regulates activation of c-Jun N-terminal kinase under oxidative stress

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ABSTRACT

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) acts as a sensor under oxidative stress, leading to induction of various biological responses. Given that mitogen-activated protein kinase (MAPK) signaling pathways mediate cellular responses to a wide variety of stimuli, including oxidative stress, here, we aimed to elucidate whether a cross-talk cascade between GAPDH and MAPKs occurs under oxidative stress. Of the three typical MAPKs investigated—extracellular signal-regulated kinase, p38, and c-Jun N-terminal kinase (JNK)—we found that hydrogen peroxide (H_2O_2)-induced JNK activation is significantly reduced in HEK293 cells treated with small-interfering (si)RNA targeting *GAPDH*. Co-immunoprecipitation with a GAPDH antibody further revealed protein–protein interactions between GAPDH and JNK in H_2O_2-stmulated cells. Notably, both JNK activation and these interactions depend on oxidation of the active-site cysteine (Cys152) in GAPDH, as demonstrated by rescue experiments with either exogenous wild-type GAPDH or the cysteine-substituted mutant (C152A) in endogenous *GAPDH*-knockdown HEK293 cells. Moreover, H_2O_2-induced translocation of Bcl-2-associated X protein (Bax) into mitochondria, which occurs downstream of JNK activation, is attenuated by endogenous *GAPDH* knockdown in HEK293 cells. These results suggest a novel role for GAPDH in the JNK signaling pathway under oxidative stress.

1. Introduction

Beyond its critical role in glycolysis, glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) has numerous non-glycolytic functions associated with a wide range of cellular processes, including DNA repair [1], membrane trafficking [2], tRNA export [3], transcription [4], heme metabolism [5], apoptosis [6], and necrosis [7]. Studies further suggest that GAPDH likely acts as an oxidant stress sensor, coordinating cellular responses to these damaging agents [8]. Under oxidative stress, such as in the presence of nitric oxide and hydrogen peroxide (H_2O_2), GAPDH translocates to the nucleus [9], where it induces p300-dependent transcription of apoptotic genes [10] and activates poly (ADP-ribose) polymerase-1 [11], ultimately resulting in epigenetic changes within cells [12]. Oxidative stress also promotes intermolecular disulfide bond formation between GAPDH monomers, leading to amyloidal GAPDH aggregation and, eventually, necrotic cell death [13]. Our lab has previously shown a critical role for the GAPDH active site cysteine-152 (Cys152) in both the nuclear translocation and aggregation of this protein [14,15]. Cys152 is easily oxidized, and serine/alanine substitutions at this site disrupt GAPDH-mediated cellular responses under oxidative stress [16].

Mitogen-activated protein kinases (MAPKs) are a highly conserved family of serine/threonine kinases, which comprise a series of signaling cascades that translate extracellular signals into cellular responses [17]. Three core MAPKs have been identified: extracellular signal-regulated kinase (ERK), p38, and c-Jun N-terminal kinase (JNK). ERK controls cell growth, proliferation, and differentiation, whereas p38 and JNK are responsive to stress stimuli, including oxidative stress, and play pivotal roles in the inflammatory and apoptotic responses, respectively [18,19]. Direct exposure of cells to exogenous H_2O_2 mimics oxidative stress and leads to activation of MAPK signaling pathways [20]. Moreover, apoptosis signal-regulating kinase-1 (ASK-1), also known as MAPK kinase 5 (MAP3K5), is involved in the activation of both p38 and JNK in response to a variety of stimuli, including H_2O_2, and may also

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Abbreviations

ANOVA	one-way analysis of variance
ASK1	apoptosis signal-regulating kinase 1
Bax	Bcl-2-associated X protein
BSA	bovine serum albumin
DMEM	Dulbecco's Modified Eagle Medium
ERK	extracellular signal-regulating kinase
FBS	fetal bovine serum
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
IP	immunoprecipitation
JNK	c-Jun N-terminal kinase
MAPKs	mitogen-activated protein kinases
PFA	paraformaldehyde
PMSF	phenylmethylsulfonyl fluoride;
PVDF	polyvinylidene difluoride;
PBS	phosphate-buffered saline;
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
siRNA	small-interfering RNA
S.E.M.	standard error of the mean
TNF-α	tumor necrosis factor-α

act as a second messenger downstream of other factors, such as tumor necrosis factor (TNF)-α [21,22].

In this study, we aimed to determine whether a crosstalk cascade between GAPDH and MAPKs occurs under oxidative stress induced by exposure to H_2O_2. We report that oxidative modification of GAPDH promotes activation of JNK specifically, but not of ERK or p38, via protein−protein interactions, and critically, this interaction modulates the mitochondrial translocation of Bcl-2-associated X protein (Bax), downstream of JNK signaling pathway activation.

2. Materials and methods

2.1. Chemicals, antibodies, and plasmids

Unless otherwise noted, all chemicals were of analytical grade. Antibodies were purchased from the following companies: mouse monoclonal anti-GAPDH antibody (MAB374) from Millipore, mouse monoclonal anti-β-actin antibody (AC15) from SIGMA, rabbit polyclonal anti-Myc antibody (A-14) and rabbit polyclonal anti-Bax antibody (N-20) from Santa Cruz Biotechnology (Dallas, TX, USA). Rabbit polyclonal anti-p38 MAPK antibody, rabbit monoclonal anti-SAPK/JNK antibody (56G8), rabbit monoclonal anti-p44/42 MAPK (ERK1/2) antibody (137F5), rabbit monoclonal anti-phospho-p38 MAPK (Thr180/Tyr182) antibody (12F8), rabbit monoclonal anti-phospho-SAPK/JNK (Thr183/Tyr185) antibody (98F2), rabbit monoclonal anti-phospho-p44/42 MAPK (Thr202/Thy204) antibody (20G11), rabbit polyclonal anti-phospho-ASK-1 (Ser967/Thr845) antibody (#3764/#3765), and horseradish peroxidase-conjugated secondary antibodies against mouse IgG or rabbit IgG were from Cell Signaling Technology (Danvers, MA, USA). Rabbit monoclonal anti-ASK-1 antibody (ab45178) was from Abcam (Cambridge, UK). Alexa488-conjugated goat polyclonal anti-mouse IgG antibody was from Invitrogen. Constructs expressing human GAPDH-WT and GAPDH-C152A were cloned into the pcDNA4-TO-Myc-HisA vector (Invitrogen) [15], and those expressing rabbit GAPDH-WT and GAPDH-C149S were cloned into the pFLAG CMV vector (SIGMA), as described previously [13].

2.2. Cell culture, RNA interference, and plasmid transfection

The human HEK293 cell line (American Type Culture Collection [ATCC]) was grown at 37 °C in 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen), supplemented with 10% (v/v) fetal bovine serum (FBS), 2-mM glutamine, and antibiotics-antimycotics (Invitrogen). Human carcinoma HeLa cells (ATCC) were maintained in DMEM, supplemented with 10% FBS, 2-mM glutamine, and antibiotics-antimycotics in the same environment. Sequences of small-interfering (si)RNAs targeting *human GAPDH* were 5′-UGGUUUACAUGUUCCAAUA-3′ and 5′-UUGAUGGUACAUGACAAGG-3' (*GAPDH* siRNA#2), and non-targeting siRNA (control siRNA) sequence was 5′-UAGCGACUAAACACAUCAA-3'. Cells in 4-cm dishes at 70−80% confluency were transfected with *GAPDH*-siRNA or control-siRNA using DharmaFECT 1 Transfection Reagent (Dharmacon), according to the manufacturer's protocols. In some experiments, cells at 80−90% confluency were transfected with plasmids encoding human GAPDH-WT or GAPDH-C152A or those encoding rabbit GAPDH-WT or GAPDH-C149S at 4-μg per dish using Lipofectamine 2000 Reagent (Invitrogen). After transfections, cells were cultured for 48 h and used for in experiments.

2.3. Measurement of total ATP content in cells

Total ATP content in cells was measured using the CellTiter-Glo Luminescent Cell Viability Assay (Promega), according to the manufacturer's protocol [23].

2.4. Western blotting and co-immunoprecipitation

HEK293 cells were treated with 3-mM H_2O_2 for the indicated time periods and lysed in ice-cold lysis buffer, containing 50-mM HEPES-KOH (pH 7.5), 150-mM NaCl, 2-mM EDTA, 2-mM EGTA, 2-mM Na_3VO_4, 50-mM NaF, 0.4% TritonX-100, 10% glycerol, 1-mM PMSF, and complete protease inhibitor cocktail (Roche), followed by centrifugation at 12,000×*g* for 15 min at 4 °C. The resultant supernatants were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), with 10% (w/v) gels, and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore). Membranes were then incubated with the indicated primary antibodies at 25 °C for 1 h or 4 °C overnight and treated with horseradish peroxidase-conjugated secondary antibodies for 1 h at 25 °C. Detection was performed using both SuperSignal West Pico Chemiluminescent Substrate (GE Healthcare Japan) and LAS3000 (FUJI-FILM).

For Co-immunoprecipitation (IP), cell lysates were incubated with 4-μg of normal mouse IgG, anti-GAPDH monoclonal antibody, or anti-Myc monoclonal antibody for 1 h on ice, followed by incubation with 10-μl of 50% slurry protein G-Sepharose for 1 h with rotation at 4 °C. Beads were washed four times with 1-ml of buffer as described above, incubated for 5 min at 100 °C in 40-μl of 2.5X sample buffer, and then subjected to western blot analysis.

2.5. Immunofluorescence analysis

HEK293 cells were cultured on cover slips and transfected with either control- or *GAPDH*-siRNA. After treatment with 3-mM H_2O_2 for 1 h and 250-nM Mito-Tracker Red (Invitrogen) for 30 min, cells were fixed with 4% paraformaldehyde (PFA)/phosphate-buffered saline (PBS) for 10 min, permeabilized with 0.1% TritonX-100/PBS for 10 min, and then blocked in 5% bovine serum albumin (BSA)/PBS for 1 h. Cover slips were incubated with primary antibody against Bax for 1 h at 25 °C, followed by the 1 h incubation with Alexa488-conjugated secondary antibody, and mounted onto slides using the Mounting Medium (Dako). Images were obtained using a

confocal scanning microscope C1si-TE2000-E (Nikon) and quantified with Image J software (v.1.51).

2.6. Statistical analysis

All data are shown as the mean ± standard error of the mean (S.E.M.) of three independent experiments. For statistical analysis, two groups or multiple groups were compared by the unpaired Student's *t*-test or Dunnet's multiple test after analysis of variance (ANOVA), with * $P < 0.05$ considered to be statistically significant.

3. Results

3.1. GAPDH is required for H_2O_2-induced JNK activation in HEK293 cells

In accordance with previous reports [12,24–29], we determined as a stimulation condition, exposure of 3 mM H_2O_2 for 1 h to HEK293 cells, in order to detect MAPKs activations clearly without cellular dysfunctions. First of all, to determine whether GAPDH affects MAPK kinase activity under oxidative stress, we measured activation of ERK, p38, and JNK in the presence of H_2O_2 in control- and *GAPDH*-siRNA-treated HEK293 cells (Fig. 1). *GAPDH* knockdown efficiency in cells was determined to be approximately 68–72% at each sampling point during the 60-min time-course of H_2O_2 exposure (Fig. 1A). Moreover, we confirmed that treatment with either control- or *GAPDH*-siRNA does not affect total ATP content in HEK293 cells (Fig. 1B), indicating the phosphorylation reactions within MAPK cascades are fully functional. Notably, whereas all three MAPKs are activated by H_2O_2—evidenced by transient increases in levels of phospho-ERK, -p38, and -JNK during the 60-min exposure in both control- and *GAPDH*-siRNA-treated cells—JNK activation is significantly reduced in *GAPDH*-siRNA-treated cells at 15, 30, and 45 min (Fig. 1C). In contrast, H_2O_2-induced activation of ASK1, which regulates JNK activation as MAPK kinase, is not affected by *GAPDH*-siRNA treatment (Supplementary Fig. 1). Together, these results suggest that GAPDH is required for H_2O_2-activation of JNK in HEK293 cells.

3.2. GAPDH and JNK interact in HEK293 cells in the presence of H_2O_2

Both GAPDH and JNK are key mediators of the cellular stress response, which have been independently reported to play roles in the response to oxidative stress [30,31]. Thus, given our above data suggesting a role for GAPDH in JNK activation, we hypothesized that GAPDH may interact with JNK at the molecular level. To test this hypothesis, we performed IP experiments with GAPDH antibody in lysates from H_2O_2-treated (3 mM, 30 min) HEK293 cells, the results of which revealed an interaction between endogenous GAPDH and JNK protein (Fig. 2A). However, purified HA-tagged human JNK1β is unable to bind purified His-tagged human GAPDH *in vitro* (data not shown), suggesting that, in cells, GAPDH likely binds to JNK indirectly, possibly via scaffolding proteins known as JNK-interacting proteins (JIPs) [32].

3.3. GAPDH–JNK interaction and downstream activation of JNK requires oxidation of the GAPDH active-site cysteine, Cys152

Under stress conditions, Cys152-oxidized GAPDH binds to other protein partners, such as the E3 ubiquitin ligase, seven in absentia homolog 1 (Siah1), in order to mediate its downstream activities [6,12]. We therefore tested whether GAPDH–JNK interaction requires oxidation of the active-site cysteine (Cys152) in GAPDH. Notably, we found that transfection of wild-type (WT) Myc-tagged GAPDH (GAPDH-WT) into HEK293 cells augments GAPDH–JNK interactions in the presence of H_2O_2, whereas a Myc-tagged GAPDH mutant with an alanine substitution at Cys152 (GAPDH-C152A) has no affect (Fig. 2B). Thus, our data indicate that GAPDH interacts with JNK under oxidative stress, and this interaction likely requires the oxidation of GAPDH Cys152.

To further confirm that oxidative modification of GAPDH is involved in JNK activation, we performed a rescue experiment in *GAPDH*-knockdown HEK293 cells, using exogenous rabbit GAPDH-WT or a −C149S mutant, which closely corresponds to human GAPDH-C152A [33]. We found that reduced levels of H_2O_2-induced JNK activation in *GAPDH*-knockdown cells (Fig. 3A and B; lane 2 and red column, respectively) are restored to near control levels in cells expressing exogenous rabbit GAPDH-WT (Fig. 3A and B; lane 3 and green column, respectively). In contrast, rabbit GAPDH-C149S is unable to rescue loss of JNK phosphorylation in these cells (Fig. 3A and B; lane 4 and blue column, respectively), indicating that the active site cysteine of GAPDH plays an essential role in JNK activation under oxidative stress.

3.4. GAPDH is required for JNK-induced mitochondrial translocation of bax under oxidative stress

Lastly, we investigated the role of GAPDH in mediating the downstream effects of JNK signaling in response to oxidative stress. The pro-apoptotic protein Bax translocates to the mitochondria following JNK activation and is a key molecule involved in the stress-induced JNK signaling pathway [34]. Here, we found that exposure to H_2O_2 (3 mM, 60 min) significantly induces mitochondrial translocation of Bax in HEK293 cells treated with control-siRNA (Fig. 4A and B; upper rows and black column, respectively). Conversely, Bax translocation is inhibited in *GAPDH*-knockdown cells (Fig. 4; lower rows and red column, respectively). Hence, our data indicate that H_2O_2-induced mitochondrial translocation of Bax is dependent on the presence of GAPDH in HEK293 cells.

4. Discussion

In the present study, our primary aim was to determine if there is a crosstalk cascade between GAPDH and MAPKs that occurs in cells under oxidative stress. Our findings reveal that in HEK293 cells, GAPDH is involved in JNK activation (Figs. 1 and 3), which occurs via (likely indirect) protein–protein interaction (Fig. 2). Moreover, this GAPDH-mediated JNK activation appears to depend on the oxidation of GAPDH Cys152 (Figs. 2 and 3) and is required for the subsequent translocation of Bax into mitochondria upon exposure to H_2O_2 stress (Fig. 4). Collectively, these findings suggest a novel role for GAPDH in activation of the JNK signaling pathway under oxidative stress.

Although a wide variety of stimuli can induce the MAPK cascade in different cell types [31], here, we used direct exposure to H_2O_2 to generate oxidative stress, given that this stimulus was previously shown to induce MAPK signaling in HEK293 cells [12,21]. We further note that a different sequence-independent siRNA targeting *GAPDH* produced nearly identical results to those reported above (siRNA#2; Supplementary Fig. 2). Moreover, we investigated the relationship between GAPDH and JNK in another model and found that treatment of HeLa cells with *GAPDH*-siRNA significantly reduces TNF-α-induced JNK activation (Supplementary Fig. 3). Thus, our data strongly suggest that GAPDH regulates JNK activation in response to cellular stress. However, further studies using distinct experimental models are needed to confirm this relationship.

Our findings further show that GAPDH initiates translocation of Bax into mitochondria via JNK activation in response to H_2O_2 stress (Fig. 4). It is well established that mitochondrial Bax induces

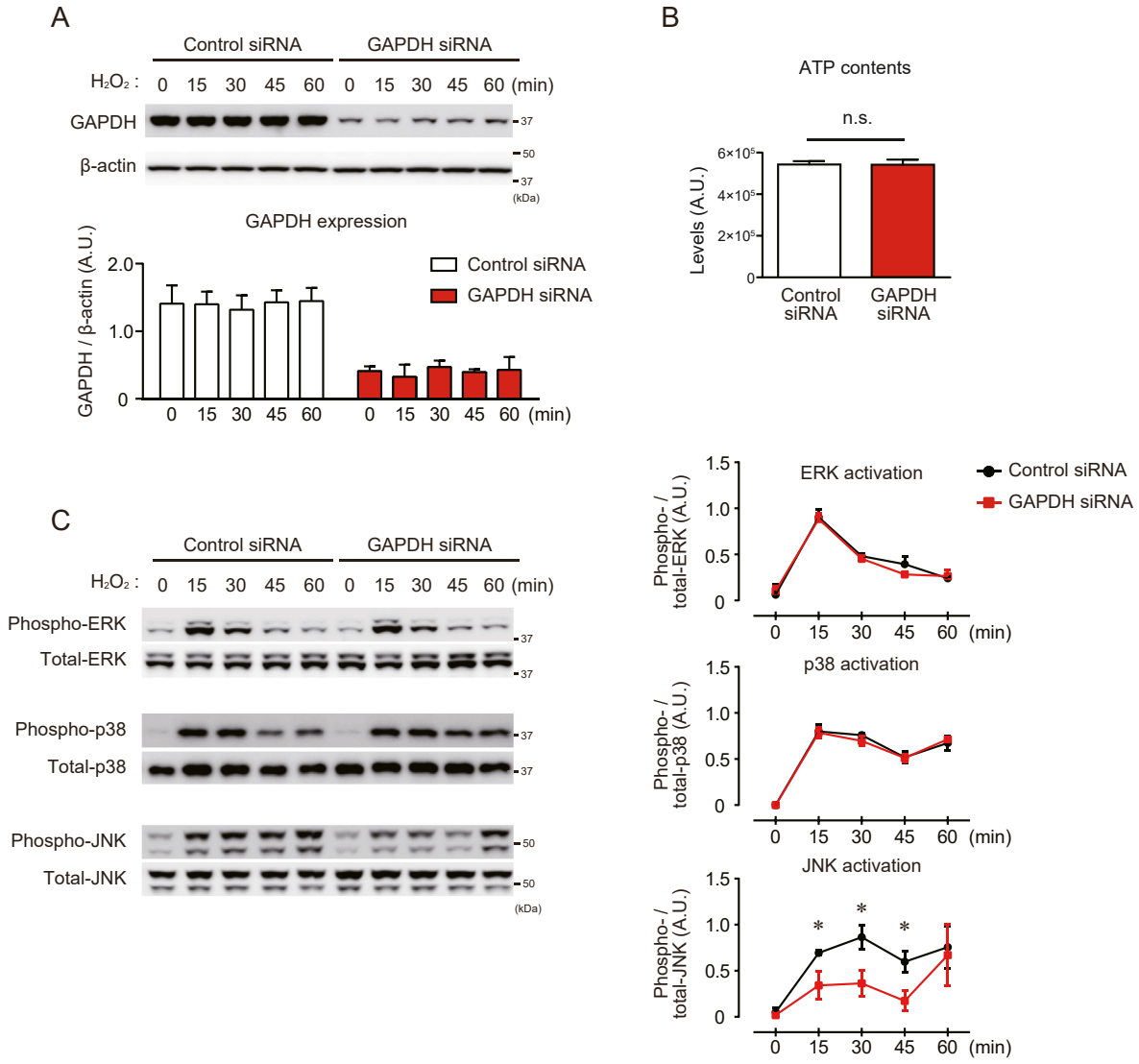

Fig. 1. Effect of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) knockdown on H_2O_2-induced mitogen activated protein kinase (MAPK) activation in HEK293 cells. (**A**) Expression levels of endogenous GAPDH in HEK293 cells treated with control small-interfering (si)RNA or *GAPDH*-siRNA and exposed to H_2O_2 for 60 min were measured by western blot. B-actin was used as the loading control. GAPDH expression levels are reduced by *GAPDH*-siRNA at the indicated timepoints. (**B**) Total ATP content in HEK293 cells treated with either control (white column) or *GAPDH*-siRNA (red column); *n. s.*, not significant. (**C**) Levels of total and phosphorylated ERK, p38, and JNK in cells treated with *GAPDH*-siRNA (red circles) or control-siRNA (black circles) and exposed to H_2O_2 were measured by western blot. B-actin was used as the loading control. Levels of JNK activation are significantly reduced by *GAPDH* knockdown at 15, 30, and 45 min $^*P < 0.05$. Data represent the means ± standard error of the mean (S.E.M.); in all cases, $n = 3$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

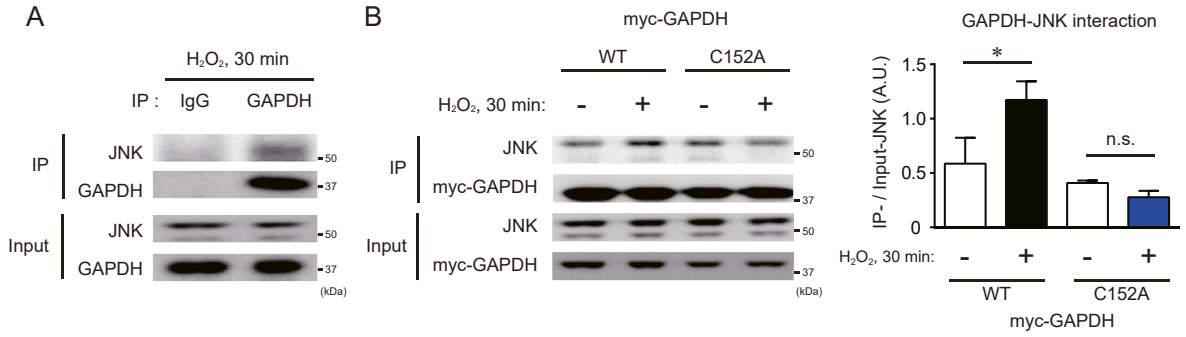

Fig. 2. GAPDH interacts with JNK in the presence of H_2O_2. (A) Co-immunoprecipitation (IP) with GAPDH antibody reveals endogenous GAPDH–JNK protein–protein interactions in H_2O_2 (3 mM, 30 min)-treated HEK293 cells. Controls with a normal mouse IgG are shown in the left lane. Input indicates the IP starting materials. (**B**) IP experiments measuring interaction between GAPDH and JNK in H_2O_2-treated HEK293 cells expressing exogenous Myc-GAPDH-wild type (WT) or the active-site mutant, Myc-GAPDH-C152A. Expression of Myc-GAPDH-WT significantly augments GAPDH–JNK protein interactions, whereas that of GAPDH-C152A does not. Data represent the means ± S.E.M. ($n = 3$). $^*P < 0.05$ compared with the control group (left white column); *n. s.*, not significant.

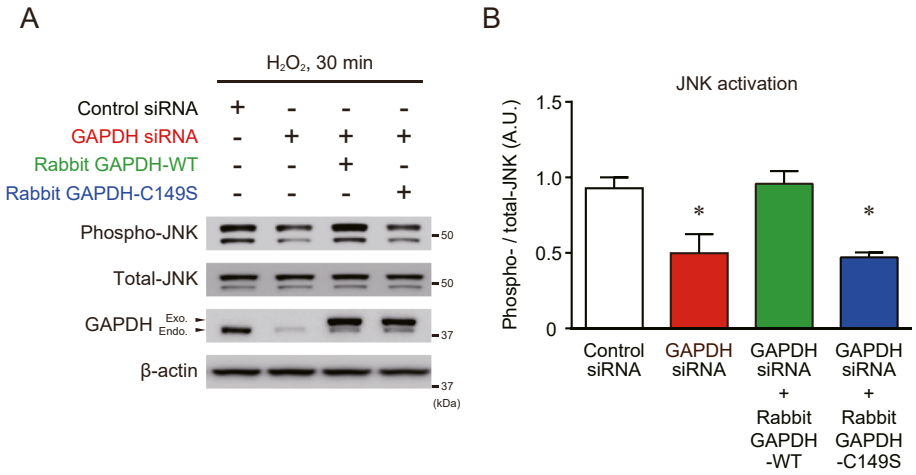

Fig. 3. Oxidative modification of GAPDH is required for the regulation of JNK activation.
(**A**) Rescue experiments with either exogenous rabbit GAPDH-WT or GAPDH-C149S mutant, which is nearly identical to human GAPDH-C152A, in endogenous *GAPDH*-knockdown HEK293 cells treated with H_2O_2. Levels of phospho-JNK and total JNK were measured by western blot; β-actin was used as the loading control; *Exo*., exogenous rabbit GAPDH-WT or GAPDH-C149S; *Endo*., endogenous human GAPDH. (**B**) The significantly decreased levels of JNK activation in *GAPDH*-siRNA-treated cells (red column) return to control levels (white column) in cells expressing exogenous rabbit GAPDH-WT (green column) but not in those expressing exogenous rabbit GAPDH-C149S (blue column). Data represent the means ± S.E.M. ($n = 3$). *$P < 0.05$ compared with the control group (left white column). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

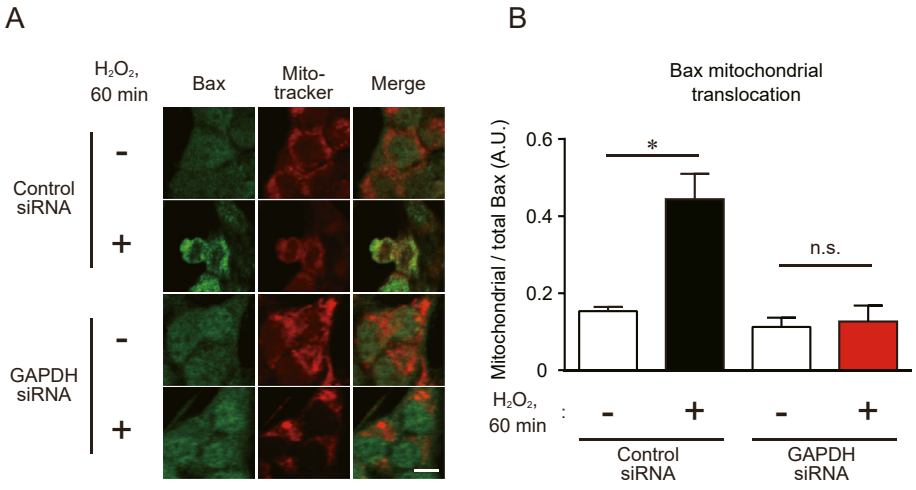

Fig. 4. GAPDH regulates JNK-mediated mitochondrial translocation of Bax induced by exposure to H_2O_2.
(**A**) Immunofluorescence staining for Bax (green) and Mito-Tracker Red (red) in HEK293 cells transfected with control- or *GAPDH*-siRNA and treated with H_2O_2. Bax-positive punctate signals in cells treated with H_2O_2 nearly colocalize with Mito-Tracker Red. Scale bar, 10 μm. (**B**) Quantification of mitochondrial (merge) per total Bax expression (green) is shown. H_2O_2-induced mitochondrial translocation of Bax (black column) is significantly attenuated by *GAPDH* knockdown (red column). Data represent the means ± S.E.M. ($n = 3$). *$P < 0.05$ compared with the control group (left white column); *n. s.*, not significant. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

cytochrome-c release, either by oligomerizing and forming a pore in the outer mitochondrial membrane [35] or by opening other channels [36], thereby inducing apoptosis [34,37]. In previous studies, we also found that oxidative stress-initiated GAPDH pathway activation leads to cellular dysfunction through either the nuclear translocation or amyloidal aggregation of GAPDH, resulting in apoptosis [6,10] or necrosis [7], respectively. Moreover, Tatton reported that increased Bax correlates with GAPDH nuclear translocation during apoptosis in the brain of Parkinson's disease [38]. In this context, GAPDH–JNK signaling might be related to the machinery underlying GAPDH nuclear translocation, but not that of its aggregation, thereby furthering our understanding for how nuclear GAPDH precisely controls biological responses under oxidative stress.

In conclusion, we here uncover a novel non-glycolytic function for GAPDH, in addition to its diverse moonlighting activities reported previously [39]. Our findings in this study may provide a new insight into underlying mechanism for GAPDH-mediated cellular dysfunctions, and reveal a possible method for combating cell damage caused by the GAPDH-dependent cell death cascade that takes place in oxidative stress-related diseases such as a Parkinson's disease.

Data availability statement

All data that support the findings of this study are available upon request from the corresponding author. All authors take responsibility for the integrity of the data and the accuracy of the data analysis.

Author contributions

H.N. and M.I. contributed to the conception and design of the study. M.I., T.K., A.K., and H.N. contributed to acquisition, analysis, and interpretation of data. H.N. contributed to writing and drafting the article, as well as critically reviewing the manuscript for important intellectual content. All authors contributed to final approval of the version to be submitted.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2023.03.044.

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