FoxO mediates APP-induced AICD-dependent cell death

X Wang¹,³, Z Wang¹,³, Y Chen¹, X Huang¹, Y Hu¹, R Zhang¹, MS Ho² and L Xue*¹

The amyloid precursor protein (APP) is a broadly expressed transmembrane protein that has a significant role in the pathogenesis of Alzheimer’s disease (AD). APP can be cleaved at multiple sites to generate a series of fragments including the amyloid β (Aβ) peptides and APP intracellular domain (AICD). Although Aβ peptides have been proposed to be the main cause of AD pathogenesis, the role of AICD has been underappreciated. Here we report that APP induces AICD-dependent cell death in Drosophila neuronal and non-neuronal tissues. Our genetic screen identified the transcription factor forkhead box O (FoxO) as a crucial downstream mediator of APP-induced cell death and locomotion defect. In mammalian cells, AICD physically interacts with FoxO in the cytoplasm, translocates with FoxO into the nucleus upon oxidative stress, and promotes FoxO-induced transcription of pro-apoptotic gene Bim. These data demonstrate that APP modulates FoxO-mediated cell death through AICD, which acts as a transcriptional co-activator of FoxO.

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Alzheimer’s disease (AD), characterized by a progressive cognitive decline leading to social and occupational debilitation, has been regarded as the most prevalent neurodegenerative disease in the world.¹ AD is pathologically differentiated from other causes of dementia by a reduction in synaptic contact,² accumulation of neurofibrillary tangles,³,⁴ aggregates of the hyper-phosphorylated microtubule-associated protein tau,⁵ and extracellular senile plaques (SPs) containing the amyloid β peptide (Aβ).⁶ Although senile individuals often develop some plaques and tangles as a consequence of aging, the brains of AD patients have a greater number of them in specific brain regions such as the temporal lobe.³

The Aβ peptide found predominantly in the SPs is generated from a large single-pass transmembrane protein termed as amyloid precursor protein (APP).⁷ Intriguingly, mutations in APP have been implicated in familial susceptibility to AD.⁸,⁹ APP can be cleaved at different sites to generate a series of fragments, including the Aβ peptides, N-APP and APP intracellular domain (AICD). The extra-cellular SPs containing the Aβ peptides have been postulated as a major cause of AD,¹⁰,¹¹ yet no direct correlation between Aβ plaques load in the brain of AD patients and the severity of clinical symptoms has been documented.¹² On the other hand, Aβ peptides were also proposed to aggregate into plaques to provide a protective mechanism by sequestering excess Aβ oligomers released from high neuronal activity.¹³ Ectopic expression of APP could induce neuronal cell death, but its mechanism has remained unclear.¹⁴ A recent study suggested that, upon deprivation of trophic factors, APP is cleaved by β-secretase and an unknown secretase to release N-APP, which binds to the death receptor 6 and induces caspase-dependent neurodegeneration.¹⁵ AICD was reported to form a transcription complex with Fe65 and histone acetyltransferase Tip60 to regulate gene expression,¹⁶ and is required for kinesin-mediated axonal transport in vivo.¹⁴ Despite many studies focusing on the pathological role of Aβ in neurodegeneration, the function of AICD in development and AD pathogenesis has remained largely elusive.

The forkhead box O (FoxO) transcription factors, initially identified at the chromosomal breakpoints in rhabdomyosarcomas and acute leukemia,¹⁷–¹⁹ contain the forkhead DNA-binding domain that belongs to the O subfamily.²⁰ The FoxO proteins have been highly conserved during evolution. Caenorhabditis elegans and Drosophila encode only one FoxO protein, named as Daf-16²¹ and dFoxO,²² respectively. Four FoxO proteins, FOXO1, FOXO2, FOXO3a, and FOXO4, have been identified in human,²³ and an additional member, FOXO6, was reported in mouse.²⁴ FoxO proteins are known to modulate various physiological processes including cell proliferation and differentiation, apoptosis, metabolism, oxidative stress, and lifespan.²²,²⁵–²⁷ Although studies have confirmed a major role of FoxO in modulating cellular response to oxidative stress, a crucial factor involved in the pathogenesis of AD, a direct connection between FoxO proteins and APP, has not been established before.

Drosophila has been previously used as an animal model to study the pathogenesis of AD, but most studies have been

¹Department of Interventional Radiology, Shanghai 10th People’s Hospital, Shanghai Key Laboratory of Signaling and Disease Research, School of Life Science and Technology, Tongji University, Shanghai, China and ²Department of Anatomy and Neurobiology, School of Medicine, Tongji University, Shanghai, China
*Corresponding author: L Xue, Department of Interventional Radiology, Shanghai 10th People’s Hospital, Shanghai Key Laboratory for Signaling and Disease Research, School of Life Science and Technology, Tongji University, 1239 Siping Road, Shanghai 200092, China. Tel/Fax: +86 21 65985407; E-mail: lei.xue@tongji.edu.cn
²These authors contributed equally to this work.

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Abbreviations: APP, amyloid precursor protein; AD, Alzheimer’s disease; Aβ, amyloid β; AICD, amyloid precursor protein intracellular domain; FoxO, forkhead box O; SP, senile plaques; APLP1, amyloid precursor-like protein 1; APLP2, amyloid precursor-like protein 2; ICD, intracellular domain; CNS, central nervous system

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focused on the neurotoxic effect of Aβ peptides. In this work, we found that expression of human APP in Drosophila induces AICD-dependent cell death and locomotion defect, both of which were mediated by the transcription factor dFoxO. This mechanism has been conserved in mammalian cells, as AICDs could physically interact with FoxO proteins and act as a transcriptional co-factor to promote FoxO-induced expression of pro-apoptotic gene Bim.

Results and Discussion

APP induces cell death in Drosophila neuronal and non-neuronal tissues. To investigate the function of APP in animal development, we first expressed human APP (APP695) in the Drosophila nervous system by the pan-neuronal elav-Gal4 driver (Figure 1b), and observed elevated cell death in the central nervous system (CNS; Figures 1c, c’, d, d’ and g). Expression of APP in the developing eye driven by GMR-Gal4 (Figure 1h) also induced strong cell death in third-instar larval eye discs (Figures 1j and m) and produced rough eyes with reduced size in the adults (Figure 1j’), as compared with the control (Figures 1i and i’). Collectively, these results indicate that ectopic expression of APP induces strong cell death in the Drosophila nervous system.

To examine whether APP also induces cell death in tissues other than the nervous system, we expressed APP in the developing thorax driven by pnr-Gal4 (Figure 1n). Compared with the control (Figures 1o and o’), expression of APP triggered cell death in the thorax region of third-instar larval wing disc (Figure 1p) and produced a small scutellum phenotype in the adult thorax (dashed line box, Figure 1p’). A similar scutellum phenotype was also observed when APP expression was driven by ap-Gal4 (Supplementary Figure S1b). In addition, expression of APP (ptc>APP) along the anterior/posterior compartment boundary in the larval wing disc (Figure 2m) induced robust cell death (Figure 2o) and produced a loss of the anterior cross vein phenotype in the adult wing (Figure 2b), as compared with the control (Figures 2a and n). Furthermore, expression of APP in the developing wing pouch driven by sd-Gal4 (sd>APP) provoked extensive cell death and generated a small wing phenotype in the adults (Supplementary Figure S2b). Together, these results demonstrate that APP is able to induce cell death in non-neuronal tissues during development.
APP induces cell death through the cleavage of AICD. APP belongs to a conserved family that includes two additional amyloid precursor-like proteins, APPL1 and APPL2, which share with APP the intracellular domain (ICD), but lack the Aβ domain. These three proteins carry out essential overlapping functions in the development, as demonstrated by phenotypes of double or triple knock-out mice. To examine whether Aβ is required for APP-induced cell death, we expressed an APP/APLP2 chimeric protein containing the ICD domain, but not the Aβ domain (Figure 1a). This chimeric protein was able to recapitulate APP-induced cell death phenotypes (Figures 1e, e', g, k, l', m, q, q', s and 2c), implying Aβ is dispensable for APP-triggered cell death. On the other hand, expression of APP\textsuperscript{ACT}, a truncated form of APP lacking AICD but retaining the Aβ region (Figure 1a), failed to induce cell death in the CNS (Figures 1f, f' and g), eye (Figure 1l, l' and m), thorax (Figures 1r, r' and s), and wing (Figures 2d and p; Supplementary Figure S2e). As this transgene expressed APP\textsuperscript{ACT} at a level comparable to that of APP or APP/APLP2\textsuperscript{4,45} and two additional APP\textsuperscript{ACT} transgenes also failed to trigger cell death (Supplementary Figure S3), we concluded that AICD is indispensable for APP-induced cell death in the development. Furthermore, expression of a dominant negative form of Presenillin, a major component of the γ-secretase complex that cleaves AICD from APP,\textsuperscript{37} suppressed APP-induced cell death (Figure 2e and data not shown), suggesting that APP induces cell death through the cleavage of AICD. Consistent with our finding, AICD, but not Aβ, is required for APP-induced blistered-wing phenotype and axonal transport defects.\textsuperscript{14,45}

APP functions through dFoxO in Drosophila. To identify the downstream factors that mediate APP-induced cell death, we performed a genetic screen in Drosophila for mutants and UAS-RNAi lines that dominantly suppress APP-induced cell death. We found that ptc>APP-induced cell death in the larval wing disc (Figure 2o) and the loss of anterior cross vein phenotype in the adult wing (Figure 2b) were significantly suppressed by mutations in the dfoxO gene (Figures 2f, k, l and q) or expression of two independent dfoxO RNAi (Figures 2g, h, k, l and r; Supplementary Figures S5g and k). The suppression of cell death is much stronger in heterozygous dfoxO\textsuperscript{194} mutants than by the expression of two dfoxO RNAi (Figures 2q, r and l; Supplementary Figure S5k), as dfoxO mRNA is more significantly reduced in heterozygous dfoxO\textsuperscript{194} mutants (Supplementary Figure S4). dfoxO\textsuperscript{194} is a recently generated null allele that removes >20 kb of the dfoxO locus and produces no dfoxO protein, and hence, is stronger than all the previously reported dfoxO alleles.\textsuperscript{38,39} Consistently, loss of dfoxO also suppressed ag>APP-induced scutellum phenotype (Supplementary Figures S1c and d) and sd>APP-induced small wing phenotype (Supplementary Figures S2c and d). On the other hand, ectopic expression of dFoxO or its human ortholog FoxO3a was able to phenocopy that of APP (Figures 2i–k and data not shown). Together, these data suggest that dFoxO is a crucial downstream factor that mediates APP-induced cell death in vivo.

AICD physically interacts with FoxO proteins in human cells. Our genetic work in Drosophila suggested that APP induces FoxO-mediated cell death through the release of AICD. To investigate the mechanism by which AICD promotes FoxO-mediated cell death, we co-expressed Myc-tagged AICD (AICD-Myc) with human FoxO1, FoxO3a, or FoxO4 in HEK 293T cells, and followed with co-immunoprecipitation assay. AICD was able to co-precipitate with human FoxO1, FoxO3a, or FoxO4 (Figure 3a), and vice versa, FoxO1, FoxO3a, or FoxO4 co-precipitated with AICD (Figure 3b), suggesting that AICD physically interacts with FoxO proteins in vivo. Consistently, AICD co-localized with FoxO predominantly in the cytoplasm of 293T cells (Figure 3e and Supplementary Figure S7). Treatment of cells with H\textsubscript{2}O\textsubscript{2} induced oxidative stress that promoted translocation of FoxO from the cytoplasm into the nucleus (Figure 3f and Supplementary Figure S7). AICD also translocated into the nucleus under oxidative stress (Figures 3c and f, Supplementary Figure S7), presumably through its interaction with FoxO proteins. In the absence of exogenous FoxO, AICD largely remained in the cytoplasm despite H\textsubscript{2}O\textsubscript{2} treatment (Figure 3c). Furthermore, AICD was able to translocate into the nucleus with FoxO4A3, a constitutive active form of FoxO4 that localized predominantly in the nucleus, without oxidative stress challenge (Figures 3d and j). Similar results for AICD and FoxO3a were also obtained in human neuroblastoma SY-SH5Y cells (Figures 3g and h). On the other hand, the predominant localization to check whether dFoxO is required for APP-induced pathological defect, we examined the larval locomotion behavior. Compared with the control larvae that moved at a speed of about 430 μm/s (Figures 2s and t; Supplementary Video 1), expression of APP in the CNS (App\textsuperscript{>1}>APP) resulted in enhanced cell death (data not shown) and considerably reduced moving ability (120 μm/s, Figures 2s and t; Supplementary Video 2). Expression of APP\textsuperscript{ACT} failed to produce such locomotion defect (data not shown), suggesting AICD is essential for the pathological function of APP. We found that APP-induced locomotion defect was significantly suppressed by a mutation in dfoxO (330 μm/s, Figures 2s and t; Supplementary Video 3) or expression of a dfoxO RNAi (270 μm/s, Figures 2s and t), which by themselves had no obvious effect on locomotion (Figures 2s and t). Together, these results indicate that dFoxO is physiologically required for APP-induced behavioral defects.
of FoxO1 and FoxO3a in the cytoplasm and FoxO4A3 in the nucleus was not affected by the presence of AICD (Supplementary Figure S8).

**AICD is a transcriptional cofactor of FoxO proteins.** Based upon the above results, we hypothesized that AICD could physically interact with FoxO proteins in the cytoplasm,
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Figure 3 Physical interaction between AICD and FoxO in human cells. (a) FoxO1, FoxO3a, or FoxO4 co-precipitated with AICD in HEK 293T cells. (b) AICD co-precipitated with FoxO3a or FoxO4 in HEK 293T cells. (c) FoxO3a enriched AICD nuclear accumulation in 293T cells under oxidative stress. A minimum of 200 cells per condition were counted. **P<0.01. (d) FoxO4A3 enriched AICD nuclear accumulation in 293T cells without oxidative stress. A minimum of 200 cells per condition were counted. ***P<0.001. (e-h) AICD co-localized with FoxO3a in 293T (e and f) or SY-SH5Y cells (g and h) before (e and g) or after (f and h) H2O2 treatment. AICD co-localized with FoxO4A3 in HEK 293T cells without oxidative stress (i). Cells were transfected with AICD-Myc and FoxO4A3 in HEK 293T cells, stained with Hoechst and incubated with anti-Myc and anti-HA antibodies. Immunoreactivity was detected with IgG conjugated to Alexa Fluor 546 (red, detects AICD), IgG conjugated to Alexa Fluor 488 (green, detects FoxO4). Co-localization was visualized by fluorescence microscope.

Figure 2 dFoxO mediates APP-induced cell death and locomotion defect in Drosophila (a–j), light images of adult wing are shown. The areas for anterior cross vein (ACV) are boxed on the left and enlarged on the right. ptc-Gal4 was used to drive the expression of GFP (a), APP (b–e–h), APP/ALPL2 (c), APP<sup>DCT</sup> (d), dFoxO (l), or human FoxO3a (j). ptc> APP induced loss-of-ACV phenotype (b) was suppressed by the expression of a dominant negative Psn (e), mutation (f), or RNAi downregulation (g, h) of dfoxO. (k) Statistical analysis of the ACV phenotype of given genotype in a–j. The percentage of adult wings with the presence of ACV is shown. Error bars means ± S.E.M., ***P<0.001. (l) Statistical analysis of acridine orange-positive cells in n–r. Error bars means ± S.E.M., ***P<0.001. (m–r) Fluorescent images of GFP expression (m) or acridine orange staining (n–r) of wing disc from 3rd instar larvae are shown. Lower panels are high magnification of boxed areas in the upper panels. ptc-Gal4 was used to drive the expression of GFP (m, n), APP (o, q, and r) or APP<sup>DCT</sup> (p). APP-induced cell death was suppressed by mutation (q) or RNAi downregulation (r) of dfoxO. (a) The larval moving track was recorded. APP expression driven by Appl-Gal4 resulted in larval locomotion defect, which was suppressed by mutation or RNAi downregulation of dfoxO. Moving speed was shown in t. Error bars means ± S.E.M., ***P<0.001. (u-x) X-Gal staining of a hid-LacZ reporter gene in 3rd instar wing discs. sd-Gal4 was used to drive the expression of GFP (u), APP (v, w), APP<sup>DCT</sup> (x). APP induced hid activation was partially suppressed in dfoxO heterozygous mutants (w). Genotypes: ptc-Gal4/UAS-GFP (a, m, and n); ptc-Gal4 UAS-APP/+ (b and o); ptc-Gal4/+; UAS-APP/ALPL2/+ (c); ptc-Gal4/UAS-APP<sup>DCT</sup> (d and p); ptc-Gal4 UAS-APP/+; UAS-Psn<sup>94</sup> (e); ptc-Gal4 UAS-APP/+; dfoxO<sup>94</sup> (f and q); ptc-Gal4 UAS-APP/+; UAS-dfoxO-IR-1/+ (g and r); ptc-Gal4 UAS-APP/+; UAS-dfoxO-IR-2/+ (h); ptc-Gal4 UAS-ALPL2 (l); ptc-Gal4/UAS-FoxO3a (j); Appl-Gal4/+; UAS-GFP/+ (left); Appl-Gal4/+; UAS-GFP/+ (2nd to left); Appl-Gal4/+; UAS-APP/+; dfoxO<sup>94</sup> (3rd to left); Appl-Gal4/+; UAS-APP/+; UAS-dfoxO-IR-1/+ (4th to left); dfoxO<sup>94</sup> (5th to left) and UAS-dfoxO-IR-1/+ (right) (i and t); sd-Gal4/+; UAS-GFP/+; hid-LacZ/+ (u); sd-Gal4/+; UAS-APP/+; hid-LacZ/+ (v); sd-Gal4/+; UAS-APP/+; hid-LacZ/dfoxO<sup>94</sup> (w); sd-Gal4/+; UAS-APP<sup>DCT</sup>/+; hid-LacZ/+(x).

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translocate with FoxO into the nucleus under oxidative stress, and function as a transcriptional co-factor of FoxO. To test this hypothesis, we examine the effect of AICD on the transcription of FoxO target genes. The pro-apoptotic gene \textit{Bim}, which encodes a BH3-only protein that directly activates the cell death machinery, is a well-known transcriptional target of FoxO proteins.\textsuperscript{43} In consistent with the hypothesis, AICD was able to upregulate \textit{Bim} promoter-mediated transcription (Figure 4a), possibly through its interaction with the endogenous FoxO proteins. Furthermore, AICD cooperated with the constitutive active FoxO4A3 to synergistically activate luciferase expression driven by \textit{Bim} promoter (Figure 4b) or 6 × DBE (Figure 4c), a synthetic promoter containing six FoxO-responsive elements.\textsuperscript{44} A similar cooperative effect with FoxO4A3 was observed for APPswe or C99 (Supplementary Figure S9) that comprises AICD, or the \(\gamma\)-secretase PS1 (Supplementary Figure S10) that promotes AICD production from endogenous APP. Hydrogen peroxide treatment of cells stably expressing C99-GFP\textsuperscript{45} resulted in enhanced AICD production accompanied with elevated \textit{Bim} expression (lane 2, Figure 4d). On the other hand, AICD production was compromised by two \(\gamma\)-secretase inhibitors, DBZ and Comp E, which resulted in significantly reduced \textit{Bim} expression (lane 4 and 5, Figure 4d). Together, these data suggested that AICD acts as a transcriptional co-factor of FoxO to activate the expression of apoptotic gene \textit{Bim}. Finally, consistent with our hypothesis, \textit{Bim} expression was upregulated in the frontal lobe of APP/PS1 double transgenic mice (Supplementary Figure S11), and in the entorhinal cortical neurons of AD patient brains.\textsuperscript{46}

**Figure 4** AICD promotes the transcription of FoxO target genes. (a) Expression of AICD-, FoxO3a-, or FoxO4-induced \textit{Bim} promoter-directed luciferase expression in HEK 293T cells. (b) and (c) AICD cooperated with FoxO4A3 to stimulate luciferase expression driven by \textit{Bim} promoter (b) or 6 × DBE (c). *\(P \leq 0.05\), **\(P \leq 0.01\), ***\(P \leq 0.001\). (d) Elevated AICD production (top) and \textit{Bim} expression (middle) upon \(H_2O_2\) challenge (lane 2, 200 \(\mu M\) for 12 h) in 293T cells stably expressing C99-EGFP (lane 1) were downregulated by pre-treatment (2 \(\mu M\) for 3 h) with \(\gamma\)-secretase inhibitor DBZ (lane 4) or Compound E (lane 5), but not by that of DMSO (lane 3). Actin was served as a loading control (bottom).
Conclusion

AD is one of the most prevalent neurodegenerative diseases in the world, although its underlying mechanism has remained largely elusive. In the present study, we analyzed the pathological functions of APP, an AD causative gene, in Drosophila, mouse and human cells. We demonstrated that ectopic expression of APP induces cell death and locomotion defect in Drosophila. Such function of APP depends on the production of AICD, and is mediated by the transcription factor dFoxO. Further studies in human cells confirmed that AICD interacts and co-localizes with FoxO proteins. Upon oxidative stress, AICD translocates from the cytoplasm into the nucleus together with FoxO, and functions as a transcriptional co-factor of FoxO to induce the transcription of Bim, a pro-apoptotic gene that activates the cell death machinery. Our findings not only expand the existing body of knowledge on the physiological function of APP, but also shed light on discovering a potential cure for AD.

Materials and Methods

Fly Stocks. Drosophila strains used include: UAS-APP, UAS-APLP2, UAS-dFoxO and UAS-FoxO3a.22,23 (UAS-Pas+/+) dFoxO14,16,18,19,20 App-Gal4, elav-Gal4, GMR-Gal4, ptc-Gal4, hid-LacZ, prs-Gal4, and sd-Gal4.45,46 UAS-GFP, UAS-dFoxO-IR-1 and UAS-dFoxO-IR-229 were previously described. Two additional UAS-APLP2 transgenic lines (#4 and #3) were obtained from the Bloomington Drosophila Stock Center at Indiana University (Bloomington, IN, USA) (#29675 and #29876).

Acridine orange staining. Brain, eye, and wing discs were dissected from the 3rd instar larvae in PBST and stained for acridine orange as described.50

X-Gal staining. Wing discs were dissected from 3rd instar larvae in PBST and stained for β-galactosidase activity as described.51

Larva locomotion test. Third instar larvae were collected and rinsed in 1% PBS, and subsequently transferred onto 3% agarose plate. The larvae were allowed to rest for 5 min before videotaping. The locomotion behaviors were analyzed by the Nikon software (NIS-Elements D, Nikon, Shanghai, China). Each genotype was tested with five larvae and each larva was tested for five times. For the track of the larvae, each genotype was recorded for 10 min and repeated for five times.

Plasmids. Expression vectors for GFP-FXXO1, GFP-FXXO3a, Flag-FXXO3a, and Bim-luciferase reporter were obtained from Z Yuan; HA-FoxO4, HA-dFoxO and D-FoxO4A3, and 6× DBE-luciferase reporter were obtained from B. Buttering.45 AICD-myc were obtained from Y Zhang,53 APPsw and C99 were obtained from N Jing.45

Cells and reagents. HEK 293T cells were maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS). SH-SYSY cells were grown in RPMI-1640 supplemented with 10% heat-inactivated FBS. Transfection was performed using FuGENE HD Transfection Reagent (Roche Applied Science, Indianapolis, IN, USA) in accordance with the manufacturer’s instructions or calcium-phosphate precipitation by standard procedure. To generate cell lines stably expressing C99-GFP, HEK293 cells were transfected with the construct and selected in the presence of 418 (500 µg/ml). HEK 293 C99-GFP cells were maintained in the medium containing 200 µg/ml of G418. Antibodies used were: anti-HA, anti-Myc, anti-FLAG, anti-APP, anti-actin, anti-GFP, EZView TM Red Anti-HA, anti-Flag M2 Affinity Gel, and protein G-Sepharose beads from Sigma (St. Louis, MO, USA); anti-FXXO1, anti-FXXO3a, anti-FXXO4, and anti-Bim from Cell Signaling (Danvers, MA, USA); Alexa Flour-488-Conjugated Goat Anti-Mouse Antibody and Alexa Flour-488-conjugated goat anti-rabbit antibody from Molecular Probes (Invitrogen, Carlsbad, CA, USA). The inhibitors of presenilin-1/secretase DBZ and Compound E (Calbiochem, San Diego, CA, USA) were used at 2 µM (final concentration).

Immunofluorescence. Cells were transfected with the indicated plasmids for 24 h and then left untreated or treated with H2O2 (200 μM, final concentration) for 1 h, fixed in 3.7% formaldehyde and permeabilized with 0.2% Triton X-100. Cells were incubated with the indicated primary antibodies followed by Alexa Fluor-conjugated secondary antibodies and observed under a fluorescence microscope (Olympus, Tokyo, Japan).

Immunoprecipitation and immunoblotting. Cells were transfected with the indicated plasmids for 24 h. Precleared lysates were incubated with the indicated antibodies followed by precipitation with protein G-Sepharose beads. Immune complexes were washed with lysis buffer, eluted in 2× SDS sample buffer, and subjected to western blot analysis using corresponding antibodies.

Luciferase reporter assay. HEK 293T cells were transfected with effector plasmids and luciferase reporters, pRL-TK Renilla luciferase construct was co-transfected as an internal control. Cells were harvested and subjected to dual-luciferase assay (Promega, Madison, WI, USA) after 24 h.

Conflict of Interest

The authors declare no conflict of interest.

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