

ORIGINAL ARTICLE

TDAG51 deficiency promotes oxidative stress-induced apoptosis through the generation of reactive oxygen species in mouse embryonic fibroblasts

Eui-Soon Park¹, Juhyeok Kim¹, Tae-uk Ha¹, Jong-Soon Choi^{2,3}, Kwan Soo Hong^{3,4} and Jaerang Rho^{1,3}

Apoptosis has an important role in maintaining tissue homeostasis in cellular stress responses such as inflammation, endoplasmic reticulum stress, and oxidative stress. T-cell death-associated gene 51 (*TDAG51*) is a member of the pleckstrin homology-like domain family and was first identified as a pro-apoptotic gene in T-cell receptor-mediated cell death. However, its pro-apoptotic function remains controversial. In this study, we investigated the role of *TDAG51* in oxidative stress-induced apoptotic cell death in mouse embryonic fibroblasts (MEFs). *TDAG51* expression was highly increased by oxidative stress responses. In response to oxidative stress, the production of intracellular reactive oxygen species was significantly enhanced in *TDAG51*-deficient MEFs, resulting in the activation of caspase-3. Thus, *TDAG51* deficiency promotes apoptotic cell death in MEFs, and these results indicate that *TDAG51* has a protective role in oxidative stress-induced cell death in MEFs.

Experimental & Molecular Medicine (2013) 45, e35; doi:10.1038/emm.2013.67; published online 9 August 2013

Keywords: apoptosis; oxidative stress; ROS; *TDAG51*

INTRODUCTION

Apoptosis has an essential role in tissue homeostasis in various organisms and is activated by a number of cellular stress responses such as oxidative stress, endoplasmic reticulum (ER) stress, and inflammatory responses.^{1–4} The receptor-mediated (extrinsic) and mitochondria-mediated (intrinsic) pathways are two major distinct apoptotic pathways that are active in mammalian cells.⁵ The receptor-mediated pathway is triggered by the stimulation of death receptors such as Fas/CD95 and the tumor necrosis factor receptor superfamily. Ligand interactions with cognate receptors initiate caspase-8 and methodically activate caspase-3. Activated caspase-3 directly induces apoptotic cell death.⁶ The mitochondria-mediated pathway is activated by several cellular stresses, including oxidative stress, ER stress, and growth factor depletion. Cellular stress induces release of cytochrome c from mitochondria, and the released cytochrome c binds to apoptotic protease activating factor-1 and caspase-9. This complex directly induces caspase-3 activation and subsequently activates apoptotic cell death.^{7,8}

Reactive oxygen species (ROS) are generated during mitochondrial oxidative metabolism, as well as during cellular responses to xenobiotics, cytokines, and bacterial invasion.⁹ The generation and accumulation of intracellular ROS can lead to intrinsic oxidative stress in cells.⁴ ROS are noxious to cells; therefore, the cell has defense mechanisms to eliminate intracellular ROS accumulation. However, an imbalance between ROS generation and elimination induces severe cellular oxidative stress. Excessive ROS derived from endogenous or exogenous routes can damage or cause complete degradation of essential molecules, including cellular lipids, proteins, and DNA, resulting in activation-induced apoptotic cell death (AICD).^{4,10} Therefore, the modulation of the oxidative stress response in cells is closely related to a series of human diseases such as neurodegeneration, atherosclerosis, diabetes, aging, and even cancer.⁹

T-cell death-associated gene 51 (*TDAG51*) was first described to have roles in the induction of *CD95/Fas* gene expression and AICD in response to the engagement of the T-cell receptor in a murine T-cell hybridoma.¹¹ This gene is

¹Department of Microbiology and Molecular Biology, College of Bioscience and Biotechnology, Chungnam National University, Daejeon, Korea; ²Division of Life Science, Korea Basic Science Institute, Daejeon, Korea; ³Graduate School of Analytical Science and Technology, Chungnam National University, Daejeon, Korea and ⁴Division of MR Research, Korea Basic Science Institute, Daejeon, Korea

Correspondence: Professor J Rho, Department of Microbiology and Molecular Biology, Chungnam National University, College of Bioscience and Biotechnology, 220 Gung-dong, Yuseong-gu, Daejeon 305 764, Republic of Korea.

E-mail: jrrho@cnu.ac.kr

Received 7 December 2012; revised 30 May 2013; accepted 11 June 2013

also known as pleckstrin homology-like domain family A member 1 (*PHLDA1*), and it possesses an N-terminal pleckstrin homology-like domain.¹² In the C-terminal region, TDAG51 contains both a proline–glutamine (PQ) repeat domain and a proline–histidine (PH) repeat domain, which may be involved in its pro-apoptotic function.^{11,13,14} However, we have previously shown that TDAG51 is not required for AICD in murine T cells in an *in vivo* study using TDAG51-deficient (*TDAG51*^{-/-}) mice.¹⁵ A human T-cell study further confirmed that TDAG51 expression is not correlated with AICD in human T cells.¹⁶ Collectively, the precise role of TDAG51 in apoptotic cell death remains controversial; therefore, it is important to elucidate whether TDAG51 expression is involved in pro-apoptotic or anti-apoptotic functions.

In the present study, we investigated the role of TDAG51 in oxidative stress-induced apoptosis in *TDAG51*^{-/-} mouse embryonic fibroblasts (MEFs). We observed that TDAG51 expression is significantly induced by oxidative stress in MEFs. Upon oxidative stress, TDAG51 deficiency results in enhanced generation of ROS in *TDAG51*^{-/-} MEFs. Thus, *TDAG51*^{-/-} MEFs were more sensitive to oxidative stress-induced apoptosis compared to wild-type (*TDAG51*^{+/+}) MEFs. These results suggest that TDAG51 deficiency promotes oxidative stress-induced apoptosis in MEFs.

MATERIALS AND METHODS

Chemicals, antibodies, and mice

Hydrogen peroxide (H₂O₂), 4',6-diamidino-2-phenylindol (DAPI), propidium iodide (PI), and 2',7'-dichlorofluorescein diacetate (DCFH-DA) were purchased from Sigma-Aldrich (St Louis, MO, USA). The following signaling inhibitors were purchased: SP600125 (c-Jun N-terminal kinase (JNK), Santa Cruz Biotechnology, Santa Cruz, CA, USA), PD98059 (extracellular signal-regulated kinase (ERK), Cell Signaling, Beverly, MA, USA), SB203580 (p38, Santa Cruz Biotechnology), and Bay11-7085 (NF-κB, Enzo Life Sciences, Farmingdale, NY, USA). The TDAG51 antibody was purchased from Santa Cruz Biotechnology. Specific antibodies against caspase-3, caspase-9, and Bax were purchased from Cell Signaling Technology. The β-actin antibody was purchased from Sigma-Aldrich. *TDAG51*^{-/-} mice were generated as previously described.¹⁵ All animal studies were approved (approval No. 10/02/09) by the Animal Experiment Ethics Committee of Chungnam National University.

MEF isolation and culture

Embryos generated from the intercross of TDAG51 heterozygous knockout (*TDAG51*^{+/-}) mice were dissected out of decidua, and all maternal tissues were removed. MEFs were isolated from each mouse embryo at embryonic day (E) 13.5. The brain and visceral organs of E13.5 embryos were surgically removed, and the remainder of the embryonic body was finely minced with sterile animal operating scissors. The minced embryos were treated repeatedly with 0.05% trypsin at 37 °C for 10 min with intermittent agitation to isolate MEFs. MEFs were maintained in Dulbecco's modified Eagle's medium (Welgene, Daegu, Korea) supplemented with 15% fetal bovine serum (Invitrogen, Carlsbad, CA, USA), 1 mM L-glutamine (Welgene), and 1 × antibiotic/antimycotic solution (Welgene) in 5% CO₂ at 37 °C. The average yield from an embryo was ~3 × 10⁷ cells. To determine

MEF genotypes, genomic DNA was extracted from MEFs, and the cells were genotyped by PCR as described previously.¹⁵

Immunoblotting assay

Total cell lysates were prepared using ice-cold lysis buffer (25 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM NaF, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 5% glycerol, and 0.5% Triton X-100). Lysates were separated by 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Amersham Biosciences, Piscataway, NJ, USA). The membrane was probed with specific antibodies, and immunoreactivity was detected using enhanced chemiluminescent reagents (Amersham Biosciences). For signaling inhibition, MEFs were treated with SP600125 (JNK, 20 μM), PD98059 (ERK, 20 μM), SB203580 (p38, 20 μM), and Bay11-7085 (NF-κB, 1 μM) with 0.5 mM H₂O₂ for 1 h, and the cell lysates were analyzed by immunoblotting as described above.

Apoptotic cell death assays

Isolated MEFs were seeded in 6-well plates (2 × 10⁵ cells ml⁻¹) and further cultured for 24 h. To induce apoptotic cell death, MEFs were then treated with the indicated concentration of H₂O₂ for 6 h. Cell survival was determined using DAPI staining and PI staining as previously described.^{17,18} For DAPI staining, the cells were washed twice with ice-cold 1 × phosphate-buffered saline (Welgene), fixed with 4% paraformaldehyde for 10 min, and then stained with 0.1 μg ml⁻¹ of DAPI (Sigma-Aldrich). Finally, the cells were observed under an inverted fluorescence microscope (DM-IRB, Leica Microsystems, Wetzlar, Germany). For PI staining, the dead cells were stained with 1 μg ml⁻¹ of PI for 1 min at room temperature and analyzed using a flow cytometer (FACSCalibur, Becton-Dickinson, San Jose, CA, USA).

Determination of generated ROS

Intracellular ROS were measured with a flow cytometer using DCFH-DA (Sigma-Aldrich) as previously described.¹⁹ Briefly, MEFs (2 × 10⁵ cells ml⁻¹) were cultured in 6-well plates. After 24 h, MEFs were incubated in serum-free culture medium without phenol red, and the cells were stimulated with 0.5 mM H₂O₂ in a time-dependent manner. After H₂O₂ stimulation, the cells were washed twice with phosphate-buffered saline and then incubated with 10 μM DCFH-DA for 30 min. Finally, the cells were harvested using cell dissociation solution (Sigma-Aldrich) and resuspended in phosphate-buffered saline containing 2% fetal bovine serum. Quantification of ROS was measured by flow cytometry using the FACSCalibur.

Construction of RNA interference expression vector and retroviral infection

TDAG51 RNA interference (*TDAG51i*) oligonucleotides (sense, 5'-GA T CCC CGT CTA CCA GGC AGA AGC ATT CAA GAG ATG CTT CTG CCT GGT AGA CTT TTT A-3'; antisense, 5'-AGC TTA AAA AGT CTA CCA GGC AGA AGC ATC TCT TGA ATG CTT CTG CCT GGT AGA CGG G-3') were synthesized and cloned into the pSuper-retro-puro (OligoEngine, Seattle, WA, USA) retroviral vector (pSR-TDAG51*i*). Green fluorescent protein (GFP) RNA interference (GFP*i*) was used as a control as described previously.²⁰ Retroviral soups harboring *TDAG51i* or GFP*i* were prepared as described previously.²⁰ Briefly, Plat.E (retrovirus packaging cell line) cells were cultured on a 60 mm dish (2 × 10⁶ cells per dish). pSR-TDAG51*i* (5 μg) or GFP*i* (5 μg) was transfected into Plat.E cells using TurboFect transfection reagent (Fermentas, Glen Burnie, MD, USA) according to the

manufacturer's instructions. At 24 h post transfection, retroviral soups harboring TDAG51i or GFPi were collected and were used to infect MEFs (1.5×10^6 cells per 60-mm dish) with $8 \mu\text{g ml}^{-1}$ polybrene (Sigma-Aldrich) for 6 h. At 6 h post infection, MEFs were selected with $2 \mu\text{g ml}^{-1}$ puromycin (Sigma-Aldrich) for 2 days. Puromycin-resistant MEFs were used for further experiments.

Luciferase reporter assays

The murine TDAG51 promoter ($P_{T51-3K-Luc}$) from -2450 to $+551$ was amplified by PCR and cloned into the pGL3-basic vector (Promega, Madison, WI, USA). A luciferase reporter assay was performed as described previously.²¹ Briefly, MEFs were plated on 24-well plates (0.5×10^5 cells per well), and the cells were co-transfected with the TDAG51 promoter plasmid and β -galactosidase expression plasmid. All transfections were performed in triplicate using TurboFect transfection reagent (Fermentas) following the manufacturer's instructions. At 48 h post transfection, MEFs were treated with $0.5 \text{ mM H}_2\text{O}_2$ for 1 h in the presence or absence of the signaling inhibitors described above. The cells were evaluated for luciferase activity using a luciferase assay kit (Promega). All values represent luciferase activity normalized to β -galactosidase activity.

Statistical analysis

All experiments were performed at least three times. Data represent the mean \pm s.d. ($n=3$ per group). Student's *t*-test was used to determine the significance of differences between experimental samples.

RESULTS

The expression of TDAG51 is enhanced by oxidative stress

TDAG51 is a known cellular stress-responsive gene,^{11,13,14} therefore, we first examined whether TDAG51 expression is enhanced by oxidative stresses in a time- and concentration-dependent manner. First, TDAG51^{+/+} MEFs were stimulated for 8 h with H_2O_2 in a dose-dependent manner. As a result, TDAG51 gene expression was greatly enhanced by H_2O_2 treatment in the concentration range between 0.5 – 0.8 mM (Figure 1a). Accordingly, the next study was designed to determine the relationship between time-dependent expression of TDAG51 and oxidative stresses. To determine TDAG51 expression during oxidative stress, TDAG51^{+/+} MEFs were stimulated with $0.5 \text{ mM H}_2\text{O}_2$ in a time-dependent manner. We observed that TDAG51 expression was significantly enhanced by H_2O_2 stimulation, and the cells were found to exhibit peak TDAG51 expression after 8 h of H_2O_2 stimulation (Figure 1b). Similarly, we detected elevated TDAG51 expression by H_2O_2 stimulation in other cell types such as Huh-7, 293T, and HeLa cells (Figure 1b). However, concordant with the results of previous reports,^{13,14} TDAG51 induction showed cell type-dependent differential expression; a rapid increase in TDAG51 expression was observed in 293T cells with a peak at 2 h, whereas a gradual increase in TDAG51 expression reached a peak at 12 h in Huh-7 and HeLa cells (Figure 1b). To further confirm the effect of oxidative stress on TDAG51 expression, we used 250 mM ethanol as another oxidative stressor to treat TDAG51^{+/+} MEFs in a time-dependent manner. Similar results were obtained demonstrating that TDAG51 expression was significantly elevated by ethanol treatment, although the

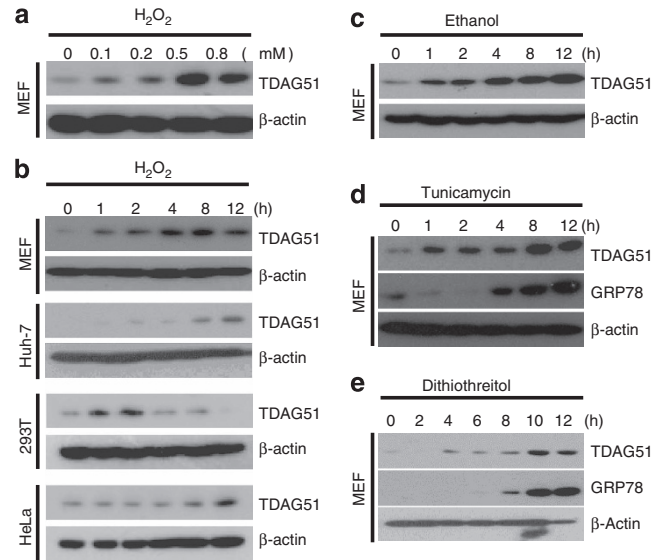


Figure 1 The expression of T-cell death-associated gene 51 (TDAG51) is enhanced by oxidative stress. (a) TDAG51 expression induced by H_2O_2 stimulation in mouse embryonic fibroblasts (MEFs). TDAG51^{+/+} MEFs were stimulated for 8 h with the indicated concentration of hydrogen peroxide (H_2O_2). TDAG51 was detected by western blot analysis with anti-TDAG51 antibody. β -Actin was used as a loading control. (b) TDAG51 expression induced by H_2O_2 stimulation in various cells. Cells were stimulated with $0.5 \text{ mM H}_2\text{O}_2$ for the indicated time periods. (c) TDAG51 expression induced by 0.25 M ethanol treatment in MEFs. (d) TDAG51 expression induced by tunicamycin ($1 \mu\text{g ml}^{-1}$) treatment in MEFs. (e) TDAG51 expression induced by $5 \text{ mM dithiothreitol}$ treatment in MEFs.

peak expression of TDAG51 was slightly delayed (Figure 1c). ER stress induced by the unfolded protein response is closely linked to the generation and accumulation of intracellular ROS, which can lead to intrinsic oxidative stress in cells.⁴ Thus, we next examined whether TDAG51 expression is enhanced by ER stress responses. Upon treatment with ER stressors such as tunicamycin and dithiothreitol, we observed that the increased expression of TDAG51 appeared to correlate with the expression of the major ER stress marker GRP78 (Figures 1d and e). These results suggest that TDAG51 is an oxidative stress-responsive gene product.

To further examine the mechanism of TDAG51 induction by oxidative stress, we generated TDAG51 promoter-reporter constructs harboring a 3 kb murine TDAG51 promoter fragment driving expression of the luciferase gene (Figure 2a). In the luciferase assays, we observed that the activity of the TDAG51 3 kb promoter ($P_{T51-3K-Luc}$) was induced in a dose-dependent manner by H_2O_2 stimulation (Figure 2a). We next determined whether mitogen-activated protein kinases and NF- κ B signaling are involved in TDAG51 induction by H_2O_2 stimulation. The promoter activities of $P_{T51-3K-Luc}$ were reduced by treatment with inhibitors of mitogen-activated protein kinases and NF- κ B signaling (Figure 2b). Coincidentally, we observed that the induction of TDAG51 protein was

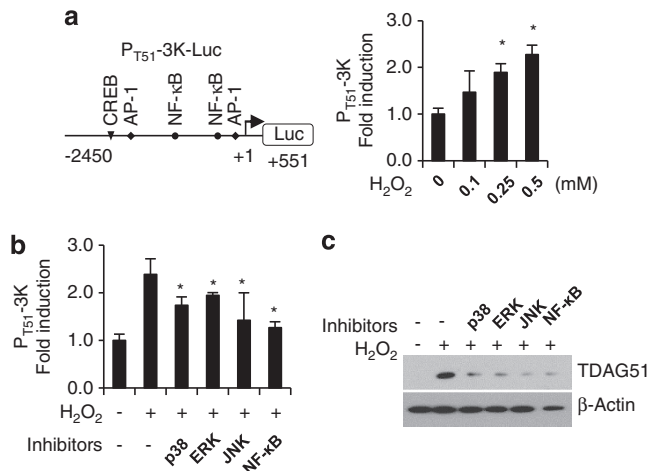


Figure 2 Gene expression of T-cell death-associated gene 51 (TDAG51) is mediated by oxidative stress-induced mitogen-activated protein (MAP) kinases and NF-κB signaling in mouse embryonic fibroblasts (MEFs). (a) The effect of hydrogen peroxide (H₂O₂) stimulation on the transcriptional activity of the TDAG51 promoter. Schematic diagram of TDAG51 promoter (P_{T51-3K-Luc}) was shown in the left panel. The nucleotide positions were numbered based on transcriptional start site, and the putative binding site of transcription factor was marked on the line. CREB, cAMP response element-binding protein; AP-1, activator protein-1. The luciferase activity of the TDAG51 promoter (P_{T51-3K-Luc}) was measured by H₂O₂ stimulation for 1 h (right). **P*<0.05. (b) The effect of inhibitors of MAP kinases and NF-κB signaling on the transcriptional activity of the TDAG51 promoter. P_{T51-3K-Luc}-transfected MEFs were treated with inhibitors and subsequently stimulated by H₂O₂ treatment (0.5 mM). **P*<0.05. (c) The effect of inhibitors of MAP kinases and NF-κB signaling on TDAG51 induction during H₂O₂ stimulation. MEFs were treated with inhibitors and subsequently stimulated by H₂O₂ treatment (0.5 mM). TDAG51 induction was analyzed by western blot analysis. β-actin was used as a loading control.

significantly reduced by treatment with inhibitors of mitogen-activated protein kinases and NF-κB signaling (Figure 2c). These results indicate that TDAG51 expression is modulated by mitogen-activated protein kinases and NF-κB signaling in response to H₂O₂ stimulation.

TDAG51 deficiency enhances intracellular ROS generation

Intracellular ROS production is dramatically increased in cellular responses to environmental stimuli.^{9,22} TDAG51 expression is involved in the oxidative stress response as shown in Figure 1; therefore, we examined whether TDAG51 deficiency affects the level of intracellular ROS production. To evaluate the effects of intracellular ROS production caused by TDAG51 deficiency, TDAG51^{-/-} MEFs were treated with 0.5 mM H₂O₂ for several time points, incubated with DCFH-DA, and then analyzed by flow cytometry. As shown in Figure 3a, intracellular ROS production in TDAG51^{-/-} MEFs was significantly enhanced by H₂O₂ stimulation compared to TDAG51^{+/+} MEFs. The level of intracellular ROS production was quantified and summarized in Figure 3b. Intracellular ROS production was significantly enhanced in a time-

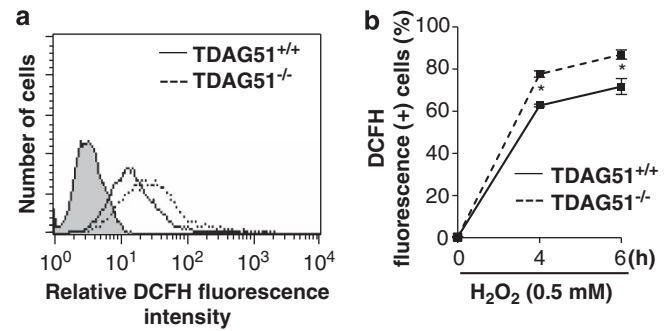


Figure 3 Enhanced intracellular reactive oxygen species (ROS) generation caused by the oxidative stress response in T-cell death-associated gene 51 deficient (TDAG51^{-/-}) mouse embryonic fibroblasts (MEFs). (a) The comparison of intracellular ROS generation between TDAG51-abundant (TDAG51^{+/+}) and TDAG51^{-/-} MEFs. MEFs were stimulated with 0.5 mM H₂O₂ for 6 h with 2',7'-dichlorofluorescein diacetate, and then the relative dichlorofluorescein (DCFH) fluorescence was analyzed by flow cytometry. (b) Summarized results of intracellular ROS production induced by H₂O₂ stimulation. All values are the mean ± s.d. for triplicate cultures. **P*<0.05.

dependent manner by H₂O₂ stimulation in TDAG51^{-/-} MEFs compared to TDAG51^{+/+} MEFs (Figure 3b). Taken together, these results indicate that TDAG51 deficiency enhances intracellular ROS production during the oxidative stress response.

Oxidative stress-induced apoptosis is elevated in TDAG51^{-/-} MEFs

Oxidative stress induces intracellular ROS accumulation in cells, which results in apoptotic cell death.^{9,22} Accordingly, we next compared apoptotic cell death between TDAG51^{+/+} and TDAG51^{-/-} MEFs induced by H₂O₂ stimulation. To test this comparison, MEFs were treated with H₂O₂ for 6 h, and cell survival was determined by DAPI staining. Cell survival was remarkably reduced 1.9- to 3.6-fold by H₂O₂ stimulation in TDAG51^{-/-} MEFs compared to TDAG51^{+/+} MEFs (Figure 4a). These results were reconfirmed by PI staining, which demonstrated that TDAG51^{-/-} MEFs were more sensitive (1.3- to 2.1-fold) to H₂O₂-induced cell death compared to TDAG51^{+/+} MEFs (Figure 4b). Similarly, cell survival decreased in a time-dependent manner after treatment with 0.5 mM H₂O₂ in TDAG51^{-/-} MEFs compared to TDAG51^{+/+} MEFs (Figure 4c). Caspase-3 activation by the apoptosome during the oxidative stress response is a final effector phase that subsequently activates apoptotic cell death.^{7,8} Oxidative stress-induced cell death was significantly enhanced in TDAG51^{-/-} MEFs as shown in Figures 4a-c; therefore, we next examined whether caspase-3 activation is enhanced by H₂O₂ stimulation in TDAG51^{-/-} MEFs. To test this idea, we treated MEFs with 0.5 mM H₂O₂ in a time-dependent manner and examined the process of caspase-3 activation by western blot analysis with a specific antibody against the active form of caspase-3. We observed that the active form of caspase-3 was significantly increased in

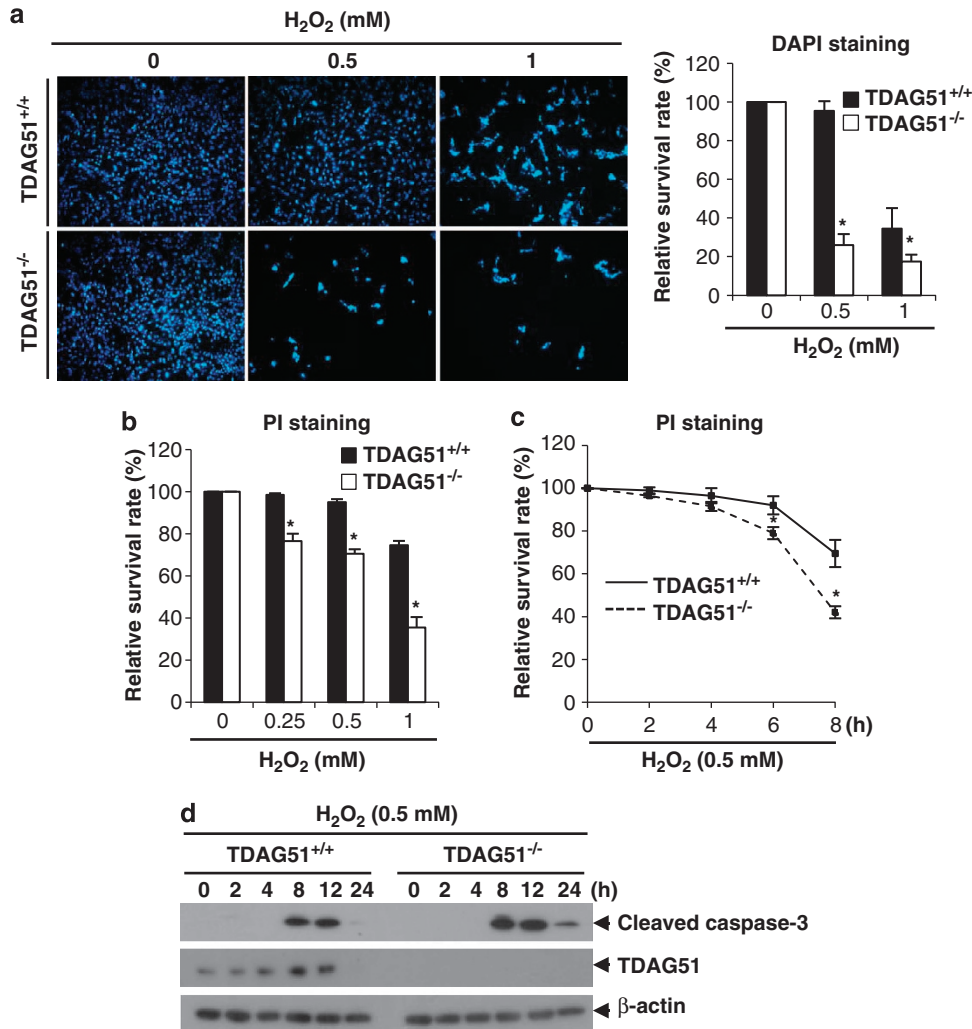


Figure 4 Oxidative stress-induced apoptosis is elevated in T-cell death-associated gene 51 deficient (TDAG51^{-/-}) mouse embryonic fibroblasts (MEFs). (a) Comparison of oxidative stress-induced apoptotic cell death between TDAG51-abundant (TDAG51^{+/+}) and TDAG51^{-/-} MEFs by 4',6-diamidino-2-phenylindol (DAPI) staining. MEFs were cultured with H₂O₂ at the indicated concentration for 10 h. The proportion of surviving cells was photographed (left) and determined (right) by DAPI staining. (b) Dose-dependent cytotoxic effects of H₂O₂ treatment on TDAG51^{-/-} MEFs were determined by propidium iodide (PI) staining. MEFs were treated with H₂O₂ at the indicated concentrations for 6 h. The proportion of surviving cells was measured by PI staining and flow cytometry. (c) Time-dependent cytotoxic effects of H₂O₂ treatment on TDAG51^{-/-} MEFs were determined by PI staining. MEFs were treated with 0.5 mM H₂O₂ for the indicated time points. The proportion of surviving cells was measured by PI staining and flow cytometry. All values are the mean ± s.d. for triplicate cultures. *P < 0.05. (d) Caspase-3 activation in TDAG51^{-/-} MEFs following H₂O₂ stimulation. MEFs were cultured with 0.5 mM H₂O₂ in a time-dependent manner. Caspase-3 activation was determined by western blot analysis with a specific antibody against cleaved caspase-3. β-Actin was used as a loading control.

TDAG51^{-/-} MEFs compared to TDAG51^{+/+} MEFs after H₂O₂ stimulation (Figure 4d). These observations indicate that TDAG51 has a protective role in oxidative stress-induced cell death.

To further examine the anti-apoptotic function of TDAG51, we investigated whether knocking down the expression of the endogenous TDAG51 gene via RNA interference (RNAi) would affect oxidative stress-induced apoptosis in MEFs. We cloned a short hairpin RNA (shRNA) specific for TDAG51 into the pSuper-retro-puro vector (pSR-TDAG51i) and performed western blot analysis to test the effects of the TDAG51 RNAi

(TDAG51i). GFPi was used as a control as described previously.²⁰ We observed that the expression of TDAG51 was significantly decreased in response to the introduction of TDAG51i into 293T cells (Figure 5a). Next, retroviral short hairpin RNA-infected MEFs were treated with H₂O₂ for 6 h, and cell survival was determined by DAPI staining. Consistent with the results in TDAG51^{-/-} MEFs (Figure 4), cell survival was reduced 1.5- to 2.7-fold by H₂O₂ stimulation in TDAG51i-transduced MEFs compared with GFPi-transduced MEFs (Figure 5b). Similarly, we observed that the active form of caspase-3 was significantly increased in TDAG51i-transduced

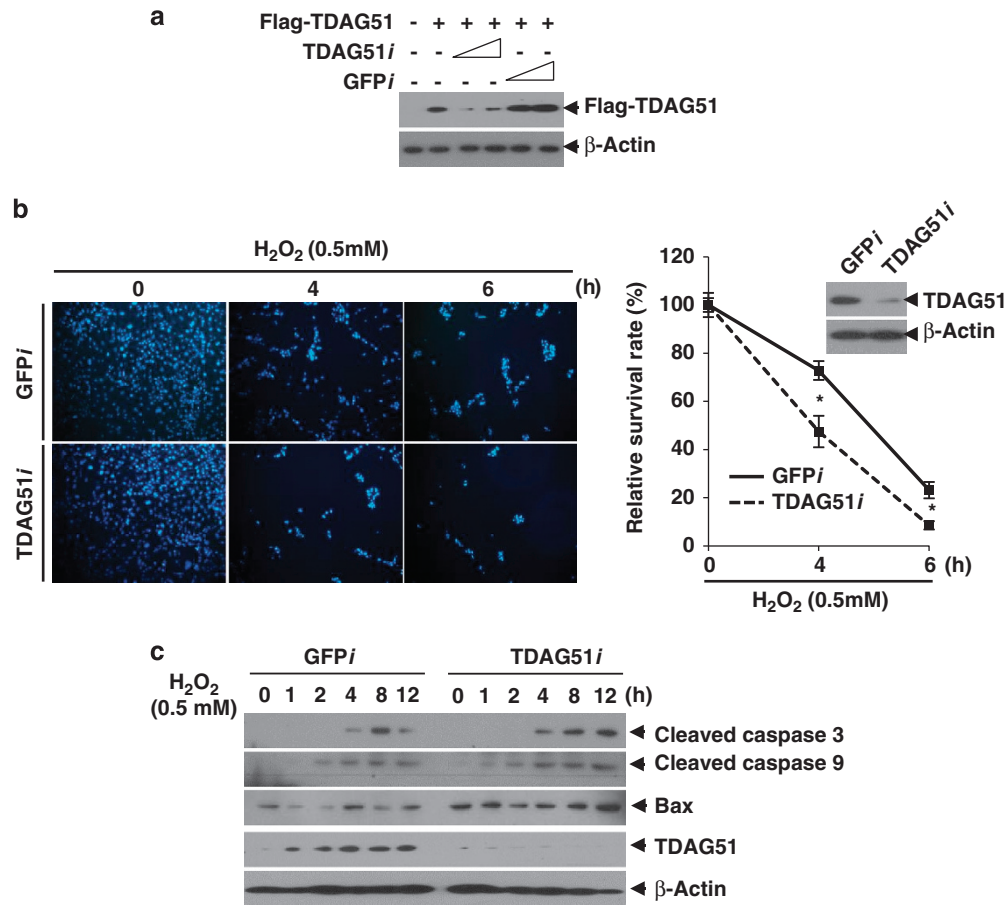


Figure 5 Oxidative stress-induced apoptosis is enhanced in T-cell death-associated gene 51 (TDAG51)-knockdown mouse embryonic fibroblasts (MEFs). **(a)** Analysis of the effects of TDAG51 knockdown by western blot analysis in 293T cells. TDAG51 knockdown was visualized by anti-Flag immunoblotting. Green fluorescent protein (GFP) RNA interference (GFP*i*) was used as a control. **(b)** Comparison of oxidative stress-induced apoptotic cell death between TDAG51 knockdown (TDAG51*i*) and control (GFP*i*) MEFs by 4',6-diamidino-2-phenylindol (DAPI) staining. MEFs were cultured with 0.5 mM H₂O₂ for 6 h. The proportion of surviving cells was photographed (left) and determined by DAPI staining (right). GFP*i* was used as a control. TDAG51 knockdown was visualized by anti-TDAG51 immunoblotting (upper right panel). **P*<0.05. **(c)** Caspase activation in TDAG51-knockdown MEFs following H₂O₂ stimulation. MEFs were cultured with 0.5 mM H₂O₂ in a time-dependent manner. The activation of apoptotic proteins was determined by western blot analysis with specific antibodies against caspase-3, caspase-9, and Bax. β-Actin was used as a loading control.

MEFs compared with GFP*i*-transduced MEFs after H₂O₂ stimulation. Furthermore, we observed that the levels of cleaved caspase-9 and Bax were increased in TDAG51*i*-transduced MEFs after H₂O₂ stimulation (Figure 5c). In summary, we conclude that oxidative stress-induced apoptosis is elevated in TDAG51^{-/-} MEFs.

DISCUSSION

Apoptosis is an elaborate cellular process that removes damaged or infected cells from normal tissue in multicellular organisms. Abnormal control of apoptotic cell death is closely related to the development of several types of human diseases such as inflammation, autoimmunity, diabetes, Alzheimer's, and cancer. Thus, understanding the regulatory process of apoptotic cell death is truly important to prevent disease development and for medical intervention. Based on these

aspects, we investigated the functional role of TDAG51 expression in oxidative stress-induced apoptotic cell death.

TDAG51 was first identified as a modulator of T-cell apoptosis through the induction of the expression of the death receptor CD95/Fas.¹¹ However, to date, several reports demonstrate that TDAG51 may have both pro- and anti-apoptotic functions depending on the cellular context and circumstances. Related to the pro-apoptotic function of TDAG51, several reports support the notion that the expression of TDAG51 is closely associated with enhanced apoptosis.^{11,23–25} Specifically, it has been reported that TDAG51 expression is highly induced by homocysteine and heat shock stress, and it promotes apoptotic cell death.^{13,14} In addition, the process of tumorigenesis is enhanced by the downregulation of TDAG51, which results in the inhibition of tumor cell apoptosis.^{26–28} However, in contrast to its pro-apoptotic function, TDAG51 has also been shown to have an

important role in insulin-like growth factor-1-induced cell survival.²⁹ Similarly, our group and another group have confirmed that TDAG51 expression is not involved in AICD in human and murine T cells.^{15,16} In addition, several groups reported that TDAG51 expression is highly enhanced in some tumor types such as pancreatic, colon, and intestinal tumors, indicating that TDAG51 may be involved in tumor cell proliferation but not in apoptotic cell death.^{16,30–32} Collectively, the exact role of TDAG51 expression in apoptotic cell death remains controversial. Thus, a better understanding of the functional role of TDAG51 in cell fate control will provide valuable insight into the regulation of tumor development and cellular stress responses.

ROS are ubiquitous, highly reactive, and short-lived derivatives of oxygen metabolism that serve as critical signaling molecules in cell proliferation and cell death.³³ ROS are generated during energy metabolism by mitochondria and from metabolic processes elsewhere in the cell that cause damage to cellular macromolecules, including nucleic acids, proteins, and lipids.⁹ Therefore, ROS production is considered to be a major cause of cellular oxidative stress.³⁴ An imbalance between ROS generation and elimination induces severe oxidative stress in cells, which results in apoptotic cell death.⁹ In this study, we demonstrated that TDAG51 is a negative modulator of apoptotic cell death induced by oxidative stress through the downregulation of ROS generation. However, an interesting question still remains involving the enhancement of ROS generation in TDAG51^{-/-} MEFs. Under physiological conditions, cellular protection against oxidative damage by ROS accumulation is provided by both enzymatic (for example, superoxide dismutase and catalase) and non-enzymatic (for example, vitamins E and C) components.³³ Interestingly, TDAG51 contains both a PQ-repeat domain and a proline-histidine-repeat domain that function as a putative transcriptional activator.¹¹ Thus, TDAG51 may be involved in the modulation of the expression of antioxidant enzymes or non-enzymatic components to eliminate excess ROS generation in response to oxidative stress. In addition, ER stress is a major source of ROS production.³⁵ Specifically, numerous clues suggest that ER stress induced by the unfolded protein response is linked to the generation of intracellular ROS, which can lead to the oxidative stress response.^{4,36} In another interesting observation, Hinz *et al.*³⁷ suggest that TDAG51 functions as a negative regulator of protein biosynthesis through an interaction with translation regulatory proteins. In our current study, we also observed that TDAG51 expression was significantly enhanced by treatment with ER stressors such as tunicamycin and dithiothreitol (Figure 1). Thus, we assume that the functional role of TDAG51 may also be involved in the regulation of the unfolded protein response in the ER. Based on this hypothesis, it is possible to explain the enhancement of ROS levels in TDAG51^{-/-} MEFs, although intensive studies are needed to reveal the mechanisms underlying a direct link between TDAG51 expression and ROS generation regulated by the unfolded protein response.

In conclusion, we investigated the role of TDAG51 in oxidative stress-induced apoptotic cell death during TDAG51 deficiency. TDAG51 expression was highly enhanced by oxidative stress responses. In response to oxidative stress, the production of intracellular ROS was significantly enhanced in TDAG51^{-/-} MEFs and resulted in the activation of caspase-3. Furthermore, TDAG51 deficiency enhanced apoptotic cell death in MEFs, and these results indicated that TDAG51 has a protective role in oxidative stress-induced cell death. Further studies will need to address the regulatory mechanism of the physiological significance of TDAG51 expression in the oxidative stress response.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This work was supported by the National Research Foundation of Korea Grant funded by the Korean Government (MSIP) (NRF-2011-0021771), Korea Basic Science Institute (D33400 and 2012-University-Institute Cooperation Program) and the Ministry of National Defense Foundation Grant (ADD: 08-10-02).

- 1 Gogna R, Madan E, Kuppusamy P, Pati U. ROS-mediated p53 core-domain modifications determine apoptotic or necrotic death in cancer cells. *Antioxid Redox Signal* 2012; **16**: 400–412.
- 2 Gorman AM, McGowan A, O'Neill C, Cotter T. Oxidative stress and apoptosis in neurodegeneration. *J Neurol Sci* 1996; **139**: Suppl 45–52.
- 3 Tabas I, Ron D. Integrating the mechanisms of apoptosis induced by endoplasmic reticulum stress. *Nat Cell Biol* 2011; **13**: 184–190.
- 4 Zhang K, Kaufman RJ. From endoplasmic-reticulum stress to the inflammatory response. *Nature* 2008; **454**: 455–462.
- 5 Fulda S, Debatin KM. Extrinsic versus intrinsic apoptosis pathways in anticancer chemotherapy. *Oncogene* 2006; **25**: 4798–4811.
- 6 Wajant H. The Fas signaling pathway: more than a paradigm. *Science* 2002; **296**: 1635–1636.
- 7 Kroemer G, Dallaporta B, Resche-Rigon M. The mitochondrial death/life regulator in apoptosis and necrosis. *Annu Rev Physiol* 1998; **60**: 619–642.
- 8 Yoshida H, Kong YY, Yoshida R, Elia AJ, Hakem A, Hakem R *et al*. Apaf1 is required for mitochondrial pathways of apoptosis and brain development. *Cell* 1998; **94**: 739–750.
- 9 Ray PD, Huang BW, Tsuji Y. Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling. *Cell Signal* 2012; **24**: 981–990.
- 10 Zhao L, Ackerman SL. Endoplasmic reticulum stress in health and disease. *Curr Opin Cell Biol* 2006; **18**: 444–452.
- 11 Park CG, Lee SY, Kandala G, Choi Y. A novel gene product that couples TCR signaling to Fas(CD95) expression in activation-induced cell death. *Immunity* 1996; **4**: 583–591.
- 12 Kuske MD, Johnson JP. Assignment of the human PHLDA1 gene to chromosome 12q15 by radiation hybrid mapping. *Cytogenet Cell Genet* 2000; **89**: 1.
- 13 Hayashida N, Inouye S, Fujimoto M, Tanaka Y, Izu H, Takaki E *et al*. A novel HSF1-mediated death pathway that is suppressed by heat shock proteins. *EMBO J* 2006; **25**: 4773–4783.
- 14 Hossain GS, van Thienen JV, Werstuck GH, Zhou J, Sood SK, Dickhout JG *et al*. TDAG51 is induced by homocysteine, promotes detachment-mediated programmed cell death, and contributes to the development of atherosclerosis in hyperhomocysteinemia. *J Biol Chem* 2003; **278**: 30317–30327.
- 15 Rho J, Gong S, Kim N, Choi Y. TDAG51 is not essential for Fas/CD95 regulation and apoptosis *in vivo*. *Mol Cell Biol* 2001; **21**: 8365–8370.
- 16 Oberg HH, Sipos B, Kalthoff H, Janssen O, Kabelitz D. Regulation of T-cell death-associated gene 51 (TDAG51) expression in human T-cells. *Cell Death Differ* 2004; **11**: 674–684.

- 17 Muppidi J, Porter M, Siegel RM. Measurement of apoptosis and other forms of cell death. *Curr Protoc Immunol* 2004; Chapter 3: Unit3: 17.
- 18 Park ES, Choi S, Kim JM, Jeong Y, Choe J, Park CS *et al*. Early embryonic lethality caused by targeted disruption of the TRAF-interacting protein (TRIP) gene. *Biochem Biophys Res Commun* 2007; **363**: 971–977.
- 19 Lu X, Kambe F, Cao X, Kozaki Y, Kaji T, Ishii T *et al*. 3beta-Hydroxysteroid-delta24 reductase is a hydrogen peroxide scavenger, protecting cells from oxidative stress-induced apoptosis. *Endocrinology* 2008; **149**: 3267–3273.
- 20 Lee SH, Kim T, Park ES, Yang S, Jeong D, Choi Y *et al*. NHE10, an osteoclast-specific member of the Na⁺/H⁺ exchanger family, regulates osteoclast differentiation and survival [corrected]. *Biochem Biophys Res Commun* 2008; **369**: 320–326.
- 21 Yu J, Choi S, Park ES, Shin B, Lee SH, Takami M *et al*. D-chiro-inositol negatively regulates the formation of multinucleated osteoclasts by down-regulating NFATc1. *J Clin Immunol* 2012; **32**: 1360–1371.
- 22 Fedoroff N. Redox regulatory mechanisms in cellular stress responses. *Ann Bot* 2006; **98**: 289–300.
- 23 Gomes I, Xiong W, Miki T, Rosner MR. A proline- and glutamine-rich protein promotes apoptosis in neuronal cells. *J Neurochem* 1999; **73**: 612–622.
- 24 Lama G, Ferraraccio F, Iaccarino F, Luongo I, Marte A, Rambaldi PF *et al*. Pelviureteral junction obstruction: correlation of renal cell apoptosis and differential renal function. *J Urol* 2003; **169**: 2335–2338.
- 25 Wang R, Zhang L, Yin D, Mufson RA, Shi Y. Protein kinase C regulates Fas (CD95/APO-1) expression. *J Immunol* 1998; **161**: 2201–2207.
- 26 Johnson EO, Chang KH, De Pablo Y, Ghosh S, Mehta R, Badve S *et al*. PHLDA1 is a crucial negative regulator and effector of Aurora A kinase in breast cancer. *J Cell Sci* 2011; **124** (Pt 16), 2711–2722.
- 27 Nagai MA, Fregnani JH, Netto MM, Brentani MM, Soares FA. Down-regulation of PHLDA1 gene expression is associated with breast cancer progression. *Breast Cancer Res Treat* 2007; **106**: 49–56.
- 28 Neef R, Kuske MA, Prols E, Johnson JP. Identification of the human PHLDA1/TDAG51 gene: down-regulation in metastatic melanoma contributes to apoptosis resistance and growth deregulation. *Cancer Res* 2002; **62**: 5920–5929.
- 29 Toyoshima Y, Karas M, Yakar S, Dupont J, Lee H, LeRoith D. TDAG51 mediates the effects of insulin-like growth factor I (IGF-I) on cell survival. *J Biol Chem* 2004; **279**: 25898–25904.
- 30 Sakthianandeswaren A, Christie M, D'Andreti C, Tsui C, Jorissen RN, Li S *et al*. PHLDA1 expression marks the putative epithelial stem cells and contributes to intestinal tumorigenesis. *Cancer Res* 2011; **71**: 3709–3719.
- 31 Segditsas S, Sieber O, Deheragoda M, East P, Rowan A, Jeffery R *et al*. Putative direct and indirect Wnt targets identified through consistent gene expression changes in APC-mutant intestinal adenomas from humans and mice. *Hum Mol Genet* 2008; **17**: 3864–3875.
- 32 Suzuki R, Miyamoto S, Yasui Y, Sugie S, Tanaka T. Global gene expression analysis of the mouse colonic mucosa treated with azoxymethane and dextran sodium sulfate. *BMC Cancer* 2007; **7**: 84.
- 33 Bondia-Pons I, Ryan L, Martinez JA. Oxidative stress and inflammation interactions in human obesity. *J Physiol Biochem* **68**: 701–711.
- 34 Hirst J, King MS, Pryde KR. The production of reactive oxygen species by complex I. *Biochem Soc Trans* 2008; **36** (Pt 5), 976–980.
- 35 Boden G. Endoplasmic reticulum stress: another link between obesity and insulin resistance/inflammation? *Diabetes* 2009; **58**: 518–519.
- 36 Ikesugi K, Yamamoto R, Mulhern ML, Shinohara T. Role of the unfolded protein response (UPR) in cataract formation. *Exp Eye Res* 2006; **83**: 508–516.
- 37 Hinz T, Flindt S, Marx A, Janssen O, Kabelitz D. Inhibition of protein synthesis by the T cell receptor-inducible human TDAG51 gene product. *Cell Signal* 2001; **13**: 345–352.



This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivs 3.0 Unported License. To view a copy of this license, visit <http://creativecommons.org/licenses/by-nc-nd/3.0/>