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CHIP has a protective role against oxidative stress-induced cell death through specific regulation of Endonuclease G

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Oxidative stress is implicated in carcinogenesis, aging, and neurodegenerative diseases. The E3 ligase C terminus of Hsc-70 interacting protein (CHIP) has a protective role against various stresses by targeting damaged proteins for proteasomal degradation, and thus maintains protein quality control. However, the detailed mechanism by which CHIP protects cells from oxidative stress has not been demonstrated. Here, we show that depletion of CHIP led to elevated Endonuclease G (EndoG) levels and enhanced cell death upon oxidative stress. In contrast, CHIP overexpression reduced EndoG levels, and resulted in reduced or no oxidative stress-induced cell death in cancer cells and primary rat cortical neurons. Under normal conditions Hsp70 mediated the interaction between EndoG and CHIP, downregulating EndoG levels in a Hsp70/proteasome-dependent manner. However, under oxidative stress Hsp70 no longer interacted with EndoG, and the stabilized EndoG translocated to the nucleus and degraded chromosomal DNA. Our data suggest that regulation of the level of EndoG by CHIP in normal conditions may determine the sensitivity to cell death upon oxidative stress. Indeed, injection of H_2O_2 into the rat brain markedly increased cell death in aged mice compared with young mice, which correlated with elevated levels of EndoG and concurrent downregulation of CHIP in aged mice. Taken together, our findings demonstrate a novel protective mechanism of CHIP against oxidative stress through regulation of EndoG, and provide an opportunity to modulate oxidative stress-induced cell death in cancer and aging.

Prolonged exposure of cells to oxidative stress results in irreversible damage and eventually in cell death. Oxidative stress is implicated in aging, carcinogenesis, and neurodegenerative diseases.[1,2] In conditions of oxidative stress, cells generate protective responses to regulate their redox state by elevating the activities of reactive oxygen species scavenger proteins and maintain protein homeostasis by eliminating damaged proteins through the ubiquitin-proteasome system. The C terminus of Hsc-70 interacting protein (CHIP) functions as an E3 ligase in protein quality control for misfolded or damaged proteins presented by Hsp70.[3,4] CHIP possesses a tetratricopeptide repeat (TPR) domain at its N terminus for interaction with Hsp70, and a U-box domain at its C terminus for interaction with E2 ubiquitin conjugating enzyme to direct the ubiquitination of substrates.[5]

Recently, CHIP has been shown to have a key role in stress recovery by ubiquitinating client proteins of Hsp70.[6] CHIP also has a protective role by targeting misfolded or damaged proteins that are associated with pathologies of the neurodegenerative diseases such as tau, a-synuclein, and expanded polyglutamine proteins.[7,8] Furthermore, CHIP deficiency decreases longevity in accelerated aging phenotypes accompanied by altered protein quality control and increased oxidative stress.[9] Collectively, these studies indicate that the protective function of CHIP against stress conditions is derived from the regulation of misfolded or damaged proteins, and overall maintenance of protein homeostasis. However, the detailed mechanism of how CHIP protects cells from specific stresses such as oxidative stress has not been elucidated.

Endonuclease G (Endo G) is a mitochondrial endonuclease that is translocated to the nucleus, where it performs caspase-independent DNA fragmentation during apoptosis.[10,11] Studies by several groups using EndoG knockout mice have not reached a unified conclusion as to whether EndoG is essential in embryogenesis and normal apoptosis,[12–14] although the involvement of EndoG in stress-induced cell death has been demonstrated in various contexts. EndoG functions in conjunction with Hsp70 to modulate heat-mediated cell death in human skin keratinocytes,[15] and EndoG mediated by BNIP3 triggers caspase-independent cell death in hippocampal neurons on oxygen–glucose deprivation[16] and ischemic cardiomyocytes.[17] Under oxidative stress, EndoG has been shown to be responsible for caspase-independent cell death in primary rat hepatocytes[18] and primary rat cortical neurons.[19]

These studies suggest that cell death due to stress conditions might be prevented by manipulating EndoG levels. On the other hand, reports that the sensitivity of certain cancer cells to chemotherapeutic drugs depends on EndoG expression[20,21] suggest that the combination of elevated EndoG and an anticancer drug might be a good strategy to enhance cancer cell death. Therefore, understanding how EndoG is regulated may have therapeutic relevance. Although EndoG has been shown to be ubiquitinated for proteasomal degradation,[22] the molecular mechanism by which EndoG is regulated remains unknown.

Here we describe a novel protective mechanism against oxidative stress that involves the regulation of EndoG by CHIP in a Hsp70- and proteasome-dependent manner.

Results

CHIP is involved in oxidative stress-induced cell death in HeLa cells.
To examine whether CHIP has a role in oxidative stress-induced cell death, HeLa cells were transfected with scrambled RNA (scRNA, control) or CHIP-targeted siRNA (siCHIP), and treated with H_2O_2. Cell death was determined by measuring DNA fragmentation using the TUNEL assay. Upon H_2O_2 treatment cell death was induced in both control cells and CHIP-depleted cells, with ~ 1.7-fold higher induction of cell death in the CHIP-depleted cells (Figure 1a). Trypan blue (TB) staining also showed similar induction of cell death in the CHIP-depleted cells (Supplementary Figure S1). Treatment of cells with the pan-caspase inhibitor Z-VAD-FMK did not significantly change the induction of cell death by H_2O_2 in both cells (Figure 1a). These results suggest that H_2O_2-induced cell death in HeLa cells occurs in a caspase-independent manner and is enhanced in the absence of CHIP. Next, we investigated the effect of overexpression of CHIP on H_2O_2-induced cell death. Following treatment with H_2O_2 bright TUNEL staining was observed in nontransfected cells or control vector-transfected cells, whereas CHIP-overexpressing cells showed weak (asterisk) or no (arrowhead) TUNEL staining (Figure 1b), suggesting that CHIP overexpression suppresses H_2O_2-induced cell death.

EndoG, a major effector of oxidative stress-induced cell death, might be regulated by CHIP.
We next investigated Endonuclease G (EndoG) and apoptosis inducing factor (AIF)[23] as candidate caspase-independent cell death effectors that might be sensitive to CHIP levels. Levels of both EndoG and AIF increased following H_2O_2 treatment regardless of CHIP expression; however, EndoG (but not AIF) levels were elevated in CHIP-depleted cells in normal conditions and upon H_2O_2 treatment (Figure 2a); therefore we focused on the role of EndoG in H_2O_2-induced cell death. We first analyzed the expression patterns of EndoG in response to H_2O_2. RT-PCR analysis showed that *EndoG* mRNA levels greatly increased within 1 h after H_2O_2 treatment (Figure 2b), and immunoblotting confirmed that EndoG protein levels also increased upon H_2O_2 treatment (Figure 2c). These data indicate that the increased expression of EndoG upon H_2O_2 treatment is due as least in part to transcriptional activation. Next, we analyzed EndoG stability following H_2O_2 treatment. The half-life of endogenous EndoG was ~ 2 h in normal conditions. EndoG was stabilized in the

presence of the proteasome inhibitor MG132, indicating that EndoG stability was regulated in a proteasome-dependent manner (Figure 2d, middle), as previously reported.[22] Interestingly, EndoG expression was stabilized or even increased over time following H_2O_2 treatment (Figure 2d, right), similar to the results of MG132 treatment (Figure 2d, middle) or H_2O_2 treatment alone without cycloheximide (Figure 2c). Together, these results indicate that the increase in EndoG levels upon H_2O_2 treatment was due not only to transcriptional induction of *EndoG* but also to inefficient degradation of EndoG by proteasomes, and further suggest that EndoG levels may be differentially regulated during oxidative stress and normal conditions.

Next, we depleted EndoG in HeLa cells to determine whether H_2O_2-induced cell death was reduced. The amount of cell death in EndoG-depleted cells following H_2O_2 treatment was $\sim 40\%$ of that in control cells (Figure 2e), revealing that EndoG is a major effector of H_2O_2-induced cell death in HeLa cells but possibly not the only one. We hypothesized that EndoG depletion might suppress the enhanced cell death observed in CHIP-depleted cells (Figure 1a). As expected, depletion of both EndoG and CHIP resulted in a level of cell death $\sim 30\%$ of that in CHIP-depleted cells and similar to that in EndoG-depleted cells (Figure 2f), suggesting a role of CHIP in the regulation of EndoG in HeLa cells. TB staining of EndoG-depleted cells or EndoG- and CHIP-double-depleted cells also showed suppression of enhanced cell death observed in CHIP-depleted cells, although it showed less reduction than the TUNEL assay results (Supplementary Figure S2A). Treatment with the potent ROS-generating agent from mitochondria, antimycin A, showed approximately twofold induction of cell death in CHIP-depleted cells, and the induced cell death was also suppressed about 55% in EndoG-depleted cells, and in EndoG- and CHIP-double-depleted cells (Supplementary Figure S2B). All together, these data suggest that CHIP might have a role in regulation of EndoG.

CHIP regulates EndoG in a Hsp70-dependent manner.
We next examined whether CHIP and EndoG physically interact. EndoG is synthesized as a pro-form that is cleaved at the N-terminus to remove the mitochondrial localization sequence (MLS) upon entering the mitochondria. Mature EndoG is released into the cytoplasm and enters the nucleus during apoptosis.[10] As we were not sure which form of EndoG might interact with CHIP, we generated GFP-EndoG WT and GFP-EndoG MLS (mature EndoG with deletion of the MLS) constructs for co-IP experiments. Both GFP-EndoG WT and GFP-EndoG MLS interacted with endogenous CHIP (Figure 3a) and Hsp70 (Figure 3b) in 293T cells, and the interactions were shown in HeLa cells (Supplementary Figures S3A and S3B). CHIP is known to bind to Hsp70, and interaction of EndoG with Hsp70 is crucial for human keratinocyte cell death.[15] Collectively, these findings suggest that Hsp70 might mediate the interaction between EndoG and CHIP. We tested this using CHIP deletion mutants (Figure 3c) and the CHIP K30A mutant that does not interact with Hsp70,[3] and showed that GFP-EndoG WT and GFP-EndoG MLS interacted with CHIP WT and the U-Box deletion mutant but not with CHIP K30A or the TPR
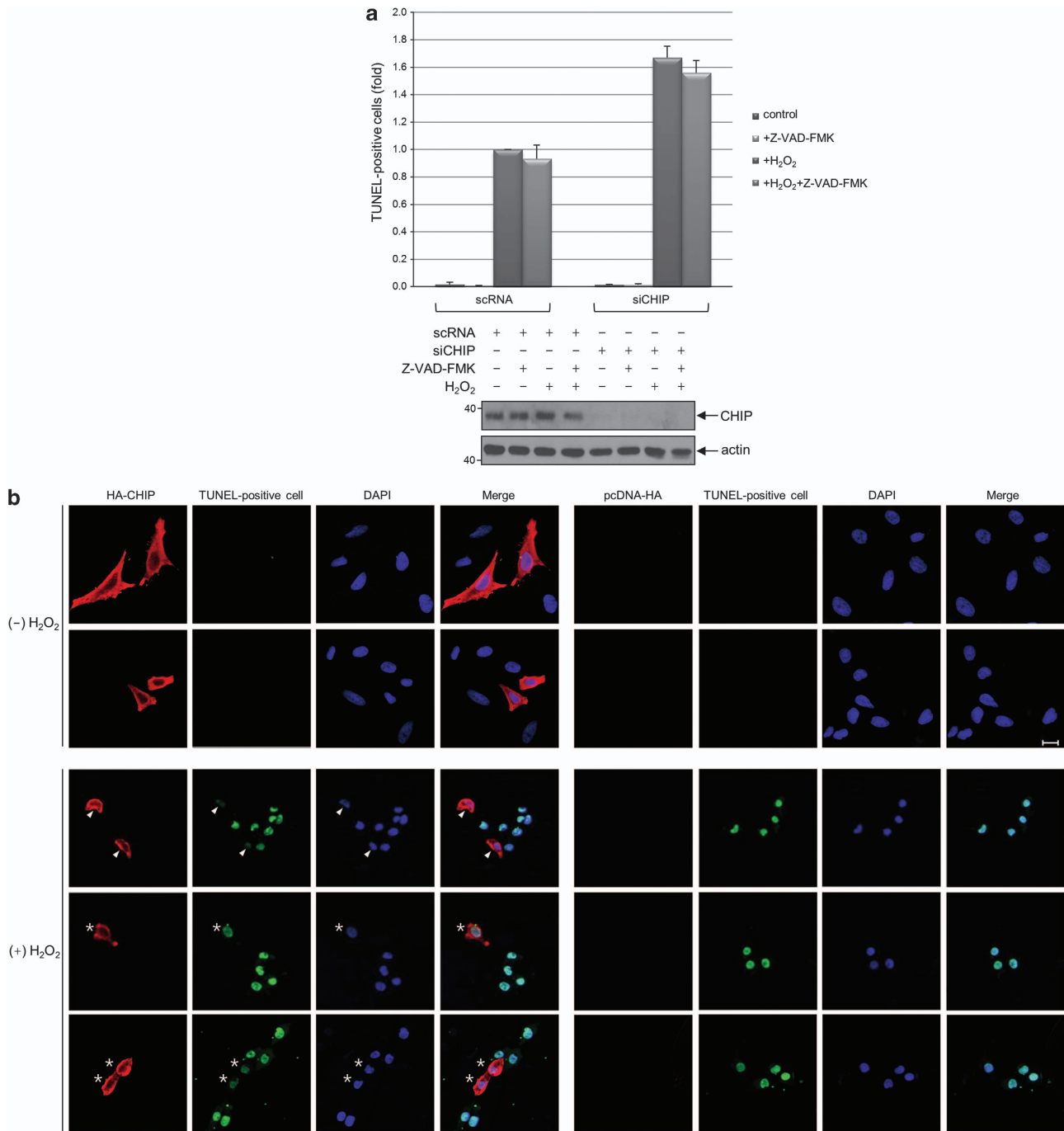
Figure 1 CHIP is involved in oxidative stress-induced cell death in HeLa cells. (**a**) H_2O_2-induced cell death in HeLa cells was caspase-independent and enhanced by depletion of CHIP. HeLa cells were transfected with scrambled RNA (scRNA) or CHIP-specific siRNA (siCHIP) for 48 h, and treated with 1 mM H_2O_2 and/or 100 μM Z-VAD-FMK for 5 h. DNA fragmentation was assessed using TUNEL assay and immunoblotting was performed with anti-CHIP and anti-actin. (**b**) Reduction of H_2O_2-induced cell death in cells that overexpress CHIP. HeLa cells were transfected with HA-CHIP or control vector for 24 h, and treated with 2 mM H_2O_2 for 5 h. Cells were immunostained with anti-HA (red) for CHIP, DAPI (blue) for nucleus, and TUNEL (green). CHIP-overexpressing cells showed no (arrowhead) or reduced (asterisk) cell death compared with control transfected cells. Bar, 20 μm

deletion mutant, identical to the interaction between Hsp70 and CHIP mutants (Figure 3d). These data demonstrate that Hsp70 mediates the interaction of EndoG with CHIP via binding to the TPR domain of CHIP.

Next, we tested whether CHIP modulates EndoG stability through ubiquitination for proteasomal degradation. EndoG WT was ubiquitinated by CHIP WT but not by CHIP K30A or the U-box deletion mutant (Figure 3e). The half-life of EndoG was much shorter in cells expressing CHIP WT than in control cells or cells expressing CHIP K30A (Figure 3f and Supplementary Figure S3C). Similar results were obtained with EndoG MLS (Supplementary Figures S3D and S3E). CHIP-depleted cells
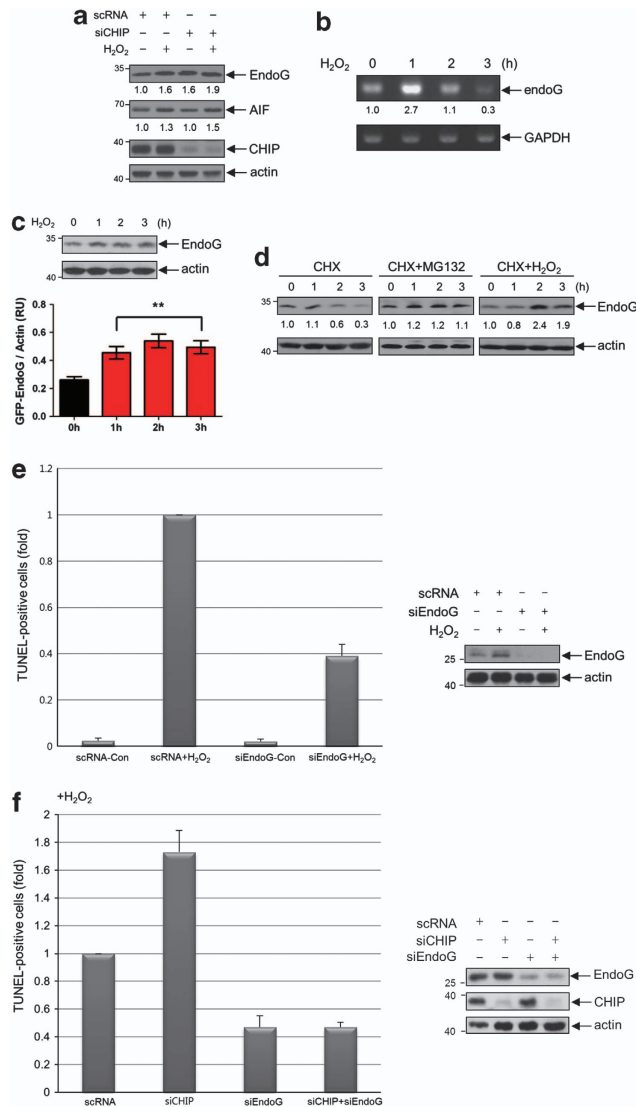
Figure 2 EndoG, a major effector of oxidative stress-induced cell death in HeLa cells, is regulated by CHIP. (**a**) EndoG levels in CHIP-depleted cells. HeLa cells were transfected with scRNA or siCHIP for 48 h, and treated with 1 mM H_2O_2 for 3 h. Cell lysates were analyzed by immunoblotting with antibody against EndoG, CHIP, AIF, and actin as indicated. (**a**, **d**) The numbers indicate relative expression of EndoG (or AIF) to actin. (**b**) Enhanced expression of *EndoG* mRNA following H_2O_2 treatment. HeLa cells were treated with 1 mM H_2O_2 for the indicated time and mRNA expression of *EndoG* was measured by RT-PCR. The relative expression of *EndoG* mRNA to GAPDH is shown. (**c**) Upregulation of EndoG protein following H_2O_2 treatment under the same experimental conditions as (**b**). Western blot analysis (top): means ± S.E.M. are given for three independent experiments, **$P < 0.01$, unpaired *t*-test (bottom). (**d**) Measurement of EndoG half-life during H_2O_2 treatment. HeLa cells were treated with cycloheximide (200 μg/ml) together with MG132 (20 μM) or H_2O_2 (1 mM) for the indicated times. EndoG protein was measured by immunoblotting. (**e**) Reduction of H_2O_2-induced cell death in EndoG-depleted cells. HeLa cells were transfected with EndoG siRNA (siEndoG) or scRNA for 48 h, and treated with 2 mM H_2O_2 for 5 h. Cell death was assessed by the TUNEL assay. (**f**) Suppression of H_2O_2-induced cell death in double-knockdown cells transfected with siCHIP and siEndoG. HeLa cells were transfected with siCHIP and/or siEndoG for 48 h, and treated with 2 mM H_2O_2 for 5 h. Cell death was measured by the TUNEL assay

contained little or no ubiquitinated GFP-EndoG, indicating that CHIP exclusively regulates EndoG levels (Figure 3g).

Hsp70 does not mediate interaction of EndoG with CHIP under oxidative stress. Under normal conditions, CHIP regulates EndoG in a proteasome-dependent manner. However, the data shown in Figure 2 revealed that EndoG was stabilized upon H_2O_2 treatment, implying that CHIP may not regulate EndoG under oxidative stress. These opposing results suggest that oxidative stress might cause changes in the interaction between EndoG and CHIP, and/or the

ubiquitination of EndoG by CHIP. The interaction of GFP-EndoG with Hsp70 was almost abolished upon H_2O_2 treatment, and concomitantly CHIP interaction with EndoG appeared to be much reduced (Figure 4a). To confirm whether EndoG interaction with CHIP was truly reduced, we performed co-IP experiments with anti-CHIP antibody. The result showed that EndoG did not interact with CHIP upon H_2O_2 treatment. In addition, the localization of EndoG was changed into the nucleus upon treatment with H_2O_2, but not Hsp70 and CHIP (Supplementary Figure S4). These results suggest that the reduced or no interaction of EndoG with
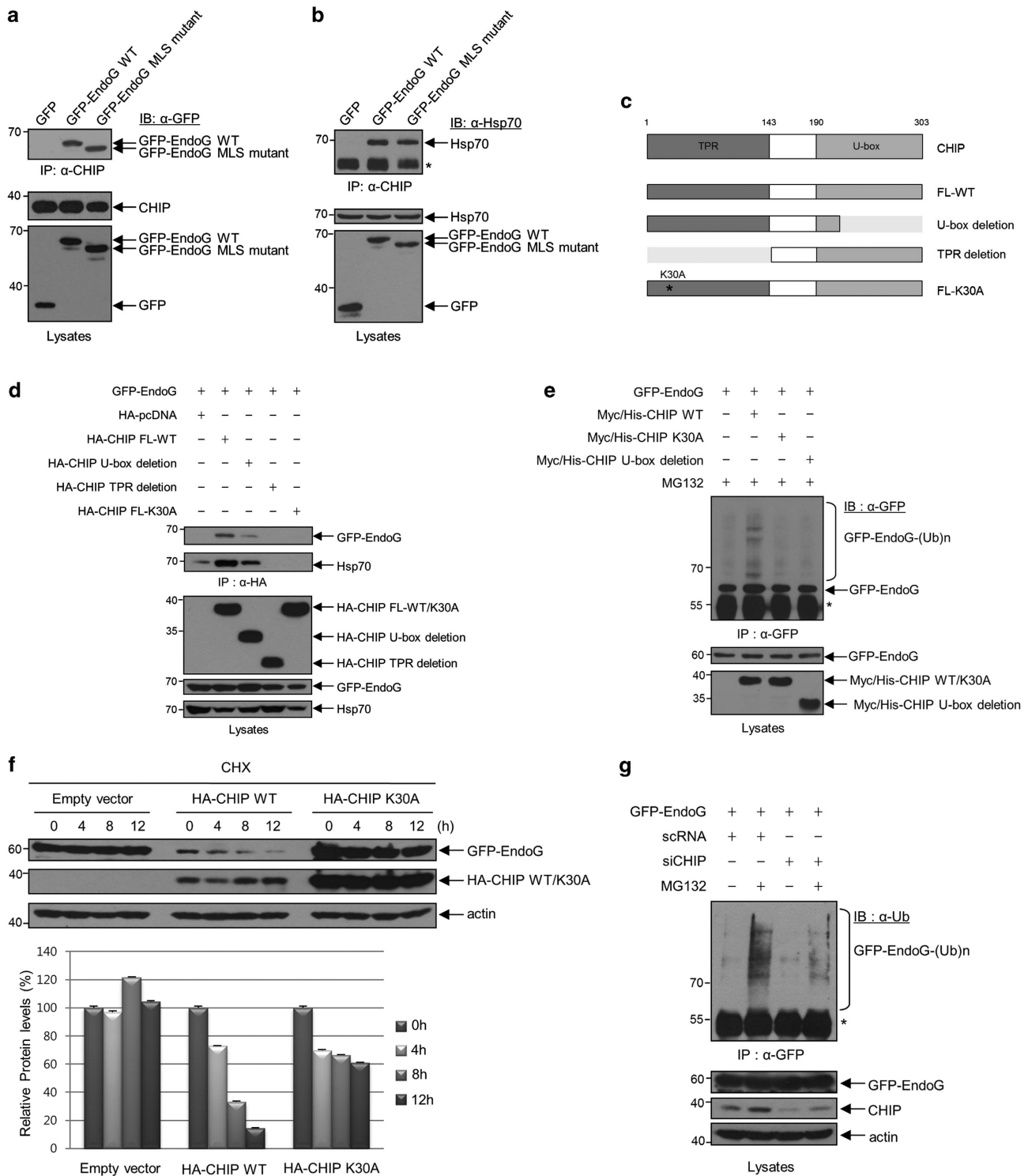
Figure 3 CHIP regulates EndoG in a Hsp70-proteasome-dependent manner. (**a**) The interaction of EndoG and CHIP. 293T cells were transfected with GFP, GFP-EndoG WT or GFP-EndoG MLS mutant for 24 h. The cell lysates were immunoprecipitated with anti-CHIP and immunoblotted with anti-GFP antibody. (**b**) Interaction of EndoG with Hsp70 under the same experimental condition as (**a**) except for IP with anti-GFP and IB with anti-Hsp70. (**c**) Schematic diagram of HA-CHIP domain mutants. (**d**) Domain analysis of CHIP for binding to EndoG. 293 cells were transfected with empty vector, HA-CHIP domain constructs, HA-CHIP K30A mutant and GFP-EndoG WT for 24 h. Cell lysates were immunoprecipitated with anti-HA and immunoblotted with anti-GFP and anti-Hsp70. (**e**) Ubiquitination of EndoG by CHIP. 293T cells were co-transfected with the indicated plasmids for 24 h and treated with the proteasome inhibitor MG132 (20 μM) for 6 h. EndoG ubiquitination was revealed by co-IP and immunoblotting with anti-GFP. *IgG. (**f**) Regulation of EndoG half-life by CHIP WT. 293T cells were co-transfected with empty vector, HA-CHIP WT or HA-CHIP K30A, and GFP-EndoG WT. After 24 h wtransfection, cells were treated with cycloheximide (CHX, 200 μg/ml) for the indicated time. EndoG protein levels were measured by immunoblotting (top) and relative protein levels (GFP-EndoG/actin) were plotted from three individual experiments (bottom). (**g**) Reduced ubiquitination of EndoG in CHIP-depleted cells. 293T cells were transfected with scRNA or siCHIP for 24 h, and transfected with GFP-EndoG WT. After 24 h transfection the cells were treated with 20 μM MG132 for 6 h. Ubiquitination was measured by IP and IB as indicated
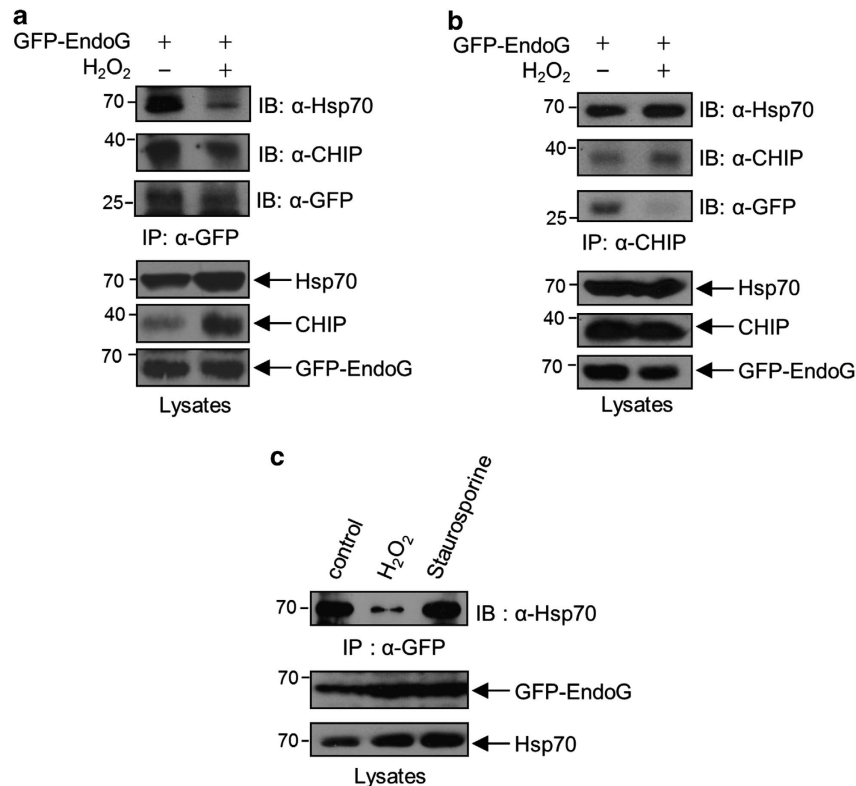
Figure 4 Hsp70 no longer mediates the interaction of EndoG with CHIP upon oxidative stress. (**a**, **b**) Loss of the interaction between EndoG and Hsp70 upon H_2O_2 treatment, even though the amount of Hsp70 in the lysate increased. HeLa cells were transfected with GFP-EndoG for 24 h and treated with 1 mM H_2O_2 for 3 h. Co-IP was performed with anti-GFP (**a**) or anti-CHIP (**b**) following by immunoblotting with anti-Hsp70, anti-CHIP, and anti-GFP. The relative expression of EndoG to actin is shown. (**c**) HeLa cells were transfected with GFP-EndoG for 24 h, and treated with 1 mM H_2O_2 for 3 h or 2 μM staurosporine for 4 h. Co-IPs were performed with anti-GFP and immunoblotting with anti-Hsp70

CHIP on H_2O_2 treatment might be due to the loss of EndoG interaction with Hsp70. However, Hsp70 showed strong interaction with CHIP following H_2O_2 treatment (Figure 4b), suggesting that the Hsp70/CHIP complex participates in protein quality control during oxidative stress. To determine whether the loss of interaction between EndoG and Hsp70 was specific to oxidative stress, we treated cells with H_2O_2, antimycin A or staurosporine, which triggers caspase-dependent cell death. Hsp70 interaction with EndoG was changed by H_2O_2 or antimycin A treatment but not by staurosporine treatment (Figure 4c and Supplementary Figure S5), suggesting that the loss of interaction between EndoG and Hsp70 was specific to oxidative stress.

Overexpression of CHIP reduces the levels of EndoG and H_2O_2-induced cell death in breast cancer cell lines. We hypothesized that the abundance of EndoG in normal conditions, which is regulated by CHIP, might determine whether cells readily undergo cell death in response to oxidative stress. To test this, we stably expressed CHIP WT and deletion mutants (Figure 3c) in the breast cancer cell line MDA-MB231, which has low levels of endogenous CHIP,[24] and examined the levels of EndoG and DNA fragmentation. As expected, EndoG levels were markedly downregulated in CHIP WT-expressing cells but were not changed or even slightly increased in cells

expressing the CHIP U-box deletion or TPR deletion mutants (Figure 5a). More importantly, upon H_2O_2 treatment CHIP WT-expressing cells showed weak or no TUNEL staining (Figure 5b, arrowheads), whereas cells expressing the deletion mutants showed very bright TUNEL staining equivalent to that of vector-transfected cells (Figure 5b), indicating that EndoG levels correlated with the incidence of oxidative stress-induced cell death. Collectively, these results support our hypothesis that the levels of EndoG in normal conditions might determine the sensitivity of breast cancer cells to cell death induced by oxidative stress.

CHIP determines the sensitivity to H_2O_2-induced cell death through regulation of EndoG in primary rat cortical neurons. As EndoG has been shown to be a critical mediator of neuronal cell death induced by oxidative stress,[19] we wondered whether CHIP suppresses oxidative stress-induced cell death in neurons through downregulation of EndoG. First, we investigated whether CHIP has a role in H_2O_2-induced cell death in primary rat cortical neurons. A TUNEL assay in the presence of Z-VAD-FMK showed that cell death was induced in both control cells and CHIP-depleted cells, with ~1.7-fold higher induction of cell death in the CHIP-depleted cells (Figure 6a). Moreover, EndoG levels were elevated in CHIP-depleted cells (Figure 6a). These data indicate that CHIP has a role in caspase-independent cell
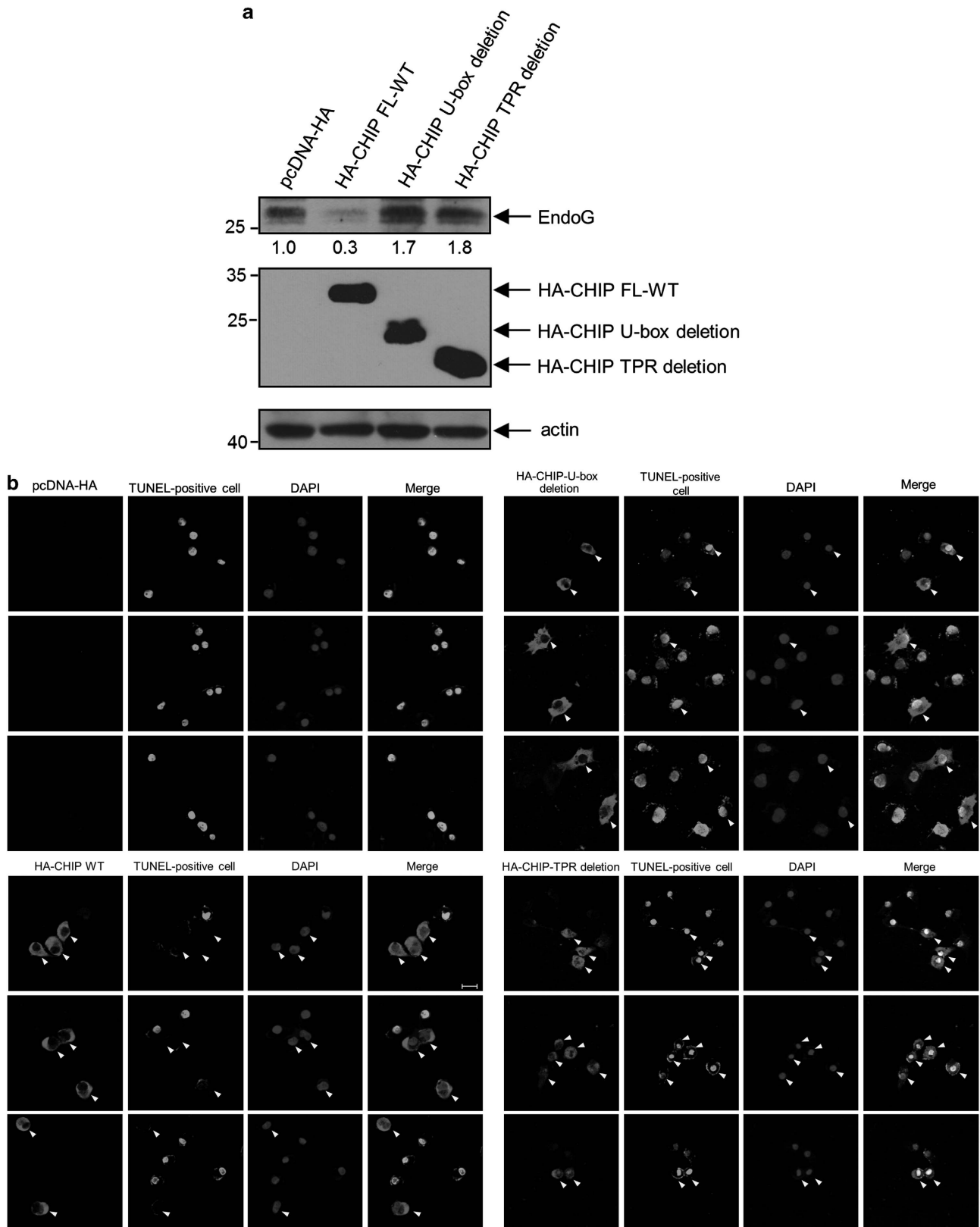
Figure 5 Overexpression of CHIP WT reduces the levels of EndoG and H₂O₂-induced cell death in breast cancer cell lines. (**a**) Regulation of EndoG by CHIP WT. HA-CHIP WT, U-box deletion, and TPR deletion mutants were transfected into MDA-MB231 cells. Proteins levels were measured by immunoblotting with anti-EndoG, anti-HA, and anti-actin antibody as indicated. The numbers indicate relative expression of EndoG to actin. (**b**) Reduced cell death in CHIP WT-overexpressing cells. MDA-MB231 cells were transfected with HA-CHIP WT and the indicated mutants for 24 h, and treated with 1 mM H_2O_2 for 5 h and immunostained with anti-HA (red), DAPI (blue) and TUNEL (green). Bar, 20 μm. Arrowheads indicate cells that overexpressed WT or mutant CHIP
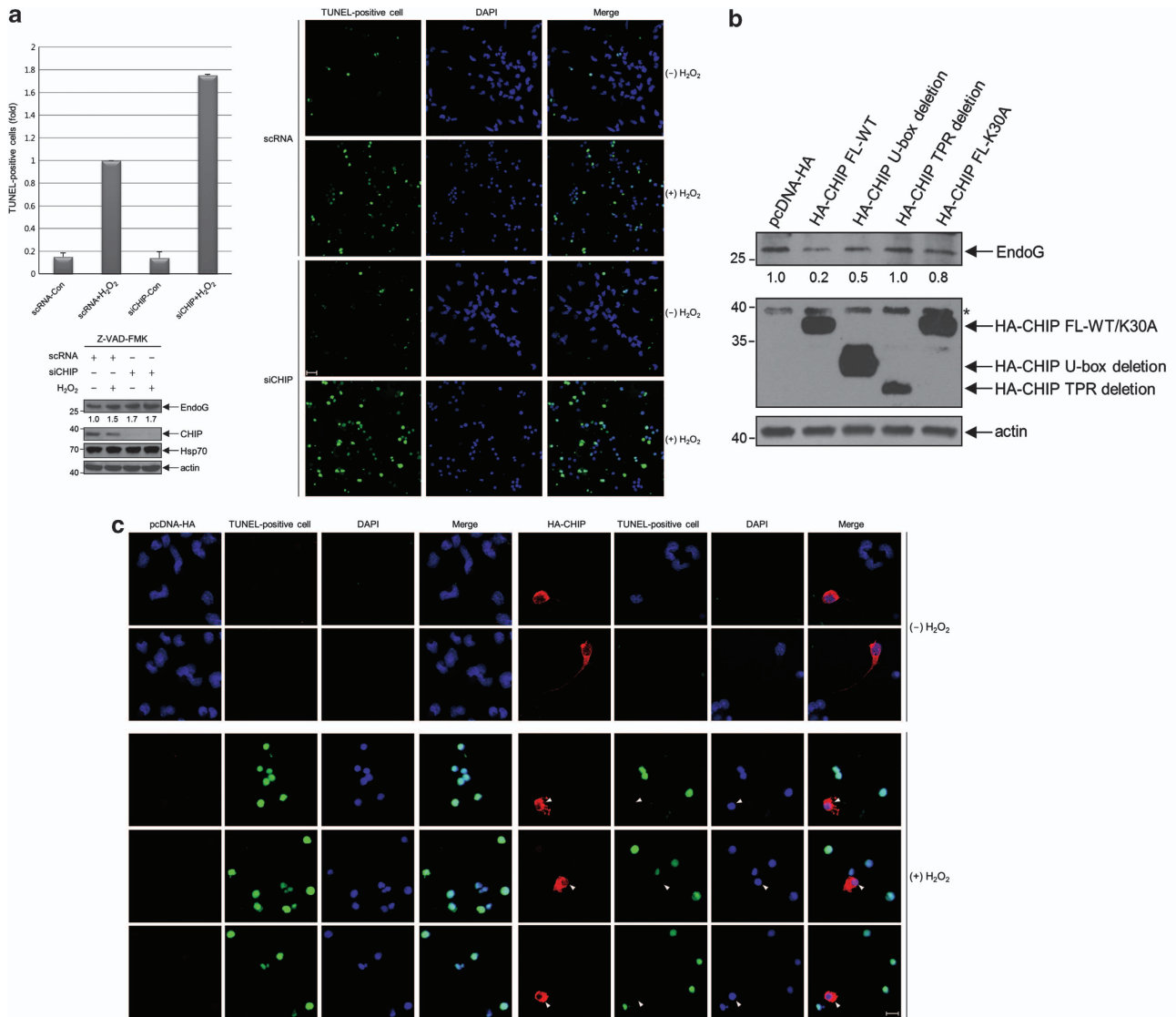
Figure 6 CHIP determines the sensitivity of primary rat cortical neurons to H_2O_2-induced cell death through regulation of EndoG. (**a**) Enhancement of H_2O_2-induced cell death in primary rat cortical neurons by CHIP siRNA. Primary rat cortical neurons were transfected with siCHIP or scRNA for 48 h, and treated with 100 μM H_2O_2 and 100 μM Z-VAD-FMK for 12 h. DNA fragmentation was assessed using TUNEL assay, and immunoblotting was performed with antibody against EndoG, CHIP, Hsp70 and actin as indicated. Bar, 20 μm. (**b**) Downregulation of EndoG by expression of CHIP WT. Primary rat cortical neurons were transiently transfected with empty vector, HA-CHIP WT or domain mutants (Figure 3c). *Nonspecific band. After 48 h transfection the proteins were visualized with anti-EndoG, anti-HA, and anti-actin antibodies. The numbers indicate relative expression of EndoG to actin. (**c**) Reduced cell death in CHIP WT-overexpressing cells. Primary rat cortical neurons were transfected with empty vector or HA-CHIP for 48 h, and treated with 100 μM H_2O_2 for 12 h. Immunostaining was performed with anti-HA (red) for CHIP and DNA fragmentation was assessed using the TUNEL assay (green). Arrowheads indicate cells that overexpress CHIP WT. Bar, 10 μm

death induced by H_2O_2 in primary rat cortical neurons, consistent with the results obtained in HeLa cells (Figure 1).

Next, we expressed CHIP WT and deletion mutants (Figure 3c) in primary rat cortical neurons, and showed that EndoG was downregulated in primary rat cortical neurons overexpressing CHIP WT but not in cells expressing the CHIP mutants (Figure 6b). Concomitantly, weakly stained or no TUNEL-positive cells were observed in rat cortical neurons expressing CHIP WT (Figure 6c, arrowheads), concordant with the results obtained in breast cancer cells (Figure 5). Thus, CHIP-modulated levels of EndoG correlated with sensitivity to oxidative stress-induced cell death in primary rat cortical neurons.

H_2O_2-induced cell death in aged mouse brain correlates with EndoG levels. Based on our data and the report that CHIP levels are downregulated in aged mice,[25] we hypothesized that EndoG levels might be elevated in aged mice. Immunoblot of whole brain lysates showed significantly higher levels of EndoG in 34-week-old mice compared with 5-week-old mice and downregulation of CHIP (Figure 7a). Next, we injected H_2O_2 or saline into each side of the hilus of the dentate gyrus (DG) of 10-week-old or 30-week-old mice and evaluated the degree of cell death. Cresyl violet staining of brain slices showed that much of the hilus of the DG in 30-week-old mice was significantly damaged following H_2O_2 injection, but not following saline injection. Quantitative
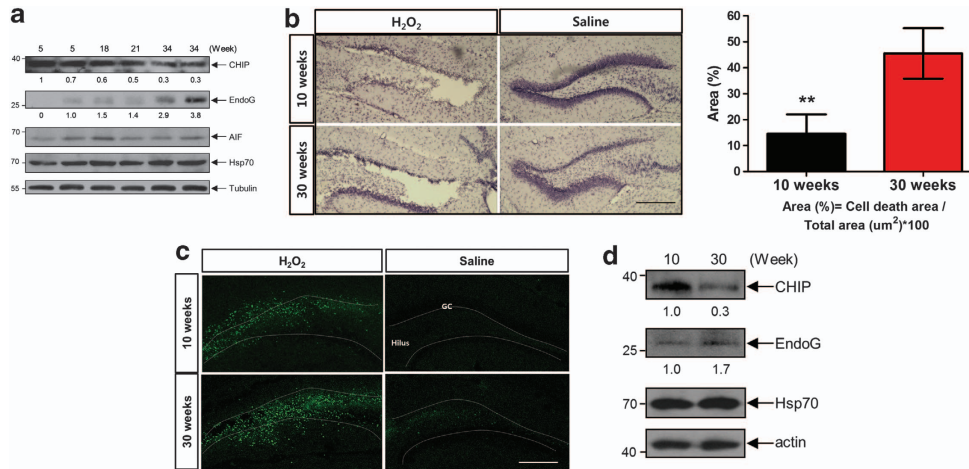
Figure 7 H_2O_2-induced cell death in the aged mouse brain correlates with the protein levels of EndoG. (**a**) Inverse correlation of EndoG and CHIP proteins in aged mouse brain. Whole brains from different aged mice (5 to 34-week-old mice and as indicated on the top) were homogenized for immunoblot analysis with anti-CHIP, anti-EndoG, anti-AIF, anti-Hsp70 and anti-tubulin antibodies. The numbers indicate relative expression of EndoG or CHIP to tubulin. (**b**, **c**) H_2O_2 and saline were injected into each side of the dentate gyrus (DG) respectively. Brain slices were collected 3 days after the injection ,and processed for cresyl violet staining or TUNEL staining. (**b**) Representative photomicrographs of cresyl violet-stained slices at bregma -1.84 and a graph showing quantitative measurement of the damaged area (Area, %) resulting from the extensive cell death induced by H_2O_2. Every fourth brain section (from bregma -1.84 to -2.20) was examined. Means $\pm$ S.E.M. are given for 10-week-old ($n = 4$) and 30-week-old mice ($n = 3$), **$P < 0.01$, unpaired *t*-test. Bar, 200 μm. (**c**) Representative photomicrograph of TUNEL staining at bregma -2.32. (**d**) Western blot analysis of the granular cell (GC) layer of the DG (age indicated on the top). The numbers indicate relative expression of EndoG or CHIP to actin. Bar, 20 μm

analysis of the damaged area showed an approximately threefold higher level of cell death in 30-week-old mice compared with 10-week-old animals (Figure 7b and Supplementary Figure S6). We performed TUNEL assays in the same experimental setting and observed brighter TUNEL staining following H_2O_2 injection in the granular cell layer (GC) of the dentate gyrus in 30-week-old mice than in that of 10-week-old mice (Figure 7c). Immunoblot of DG lysates showed significantly lower levels of CHIP and higher levels of EndoG in 30-week-old mice compared with 10-week-old mice (Figure 7d), which is consistent with WB result of whole brain lysates (Figure 7a). A relative increase in Hsp70 was observed in aged mice but it did not seem to affect EndoG levels, probably because of CHIP down-regulation in these animals.

Discussion

EndoG, an effector of oxidative stress-induced cell death, is specifically regulated by CHIP in cancer cells and primary rat cortical neurons. Recent studies have revealed that EndoG is an important effector of caspase-independent cell death in stress conditions[15,18,26,27] in spite of great debate over the role of EndoG in embryogenesis and normal apoptosis.[12–14] In the present study we found that EndoG levels increased under oxidative stress as a result of both transcriptional upregulation and inefficient regulation of protein stability. More importantly, intracellular levels of EndoG determined sensitivity to caspase-independent cell death under conditions of oxidative stress in cancer cells and primary rat cortical neurons. Furthermore, cells from the brains of old mice were more susceptible to death induced by oxidative stress and showed elevated levels of EndoG compared with brain cells of young mice. We conclude that

EndoG functions as a major effector in caspase-independent cell death induced by oxidative stress.

Our study identified CHIP as an E3 ligase for EndoG. The supporting evidence is as follows: (1) EndoG stability was regulated by CHIP via the ubiquitin–proteasome system; (2) EndoG ubiquitination was diminished in CHIP-depleted cells; (3) CHIP expression downregulated EndoG levels in breast cancer cells and primary rat cortical neurons; (4) elevated EndoG levels in the aged mouse brain are inversely correlated with CHIP levels. We propose that under normal conditions CHIP is bound to EndoG through Hsp70 and regulates EndoG levels though proteasomal degradation. Under conditions of oxidative stress EndoG no longer interacts with Hsp70 and CHIP and is translocated to the nucleus where it performs DNA fragmentation. Interestingly, the loss of EndoG interaction with Hsp70 appears to be specific to oxidative stress. Cellular proteins undergo various forms of post-translational protein modification during oxidative stress,[28,29] and oxidation of proteins influences various cellular functions due to disruption of proper protein–protein interaction.[30–32] We have tried to detect oxidation of either EndoG or Hsp70 using an oxiblot assay (Millipore, Billerica, MA, USA), but our attempts to date have failed. The detailed mechanism by which the interaction between EndoG and Hsp70/CHIP is disrupted in conditions of oxidative stress therefore remains to be elucidated.

Hsp70 as a specific mediator for EndoG regulation by CHIP. Hsp70 was previously shown to have a cytoprotective role against various stresses.[33,34] With regard to EndoG, it has been shown that Hsp70 interacts with EndoG and inhibits its DNA fragmentation activity *in vitro*,[35] and that EndoG-dependent cell death in human skin keratinocytes is modulated by Hsp70 expression upon heat stress.[15] These

observations suggest that the cytoprotective mechanism of Hsp70 against various forms of stress-induced cell death might involve suppression of EndoG activity via direct binding. However, the present study describes a novel aspect of the cytoprotective role of Hsp70, in which it functions in conjunction with CHIP against oxidative stress-induced cell death. Hsp70 appears to mediate the interaction of EndoG with CHIP by binding to both proteins, and thus mediates the subsequent regulation of EndoG by CHIP. EndoG is not downregulated without Hsp70 binding, and the resultant elevation in EndoG significantly increases the incidence of oxidative stress-induced cell death (Figures 3 and 5). Therefore, Hsp70-mediated regulation of EndoG by CHIP maintains intracellular EndoG at levels suitable for its normal function and prevents cells from excessive cell death in response to oxidative stress. A similar regulatory mechanism by Hsp90 and CHIP has been reported. Hsp90 interacts and protects thiol-modified SENP3 under mild oxidative stress, while unmodified SENP3 is maintained at low level by CHIP-dependent regulation in normal condition.[36] Hsp70/or Hsp90/CHIP complex might have a common mechanism to regulate client proteins in response to dynamic redox state in cells, which involves oxidative stress-induced post-translational modification on client proteins, and subsequent change in the interaction of Hsp70 or Hsp90 with client proteins.

As a co-chaperone E3 ligase, CHIP ubiquitinates misfolded or abnormal proteins that are bound to a molecular chaperone for proteasomal degradation during protein quality control.[6,37] Recent reports have demonstrated that CHIP specifically regulates the level of proteins such as NQO1,[25] c-Myc,[38] and PTEN[39] that are presented by Hsp70. Our study provides evidence that EndoG is specifically recognized by Hsp70 and degraded. Collectively, these reports and our data imply that Hsp70 functions as a specific mediator to present a substrate to CHIP rather than as a general chaperone to assist proper folding of a substrate. In this regard, it is highly possible that another mediator may replace Hsp70 to assist CHIP in the specific regulation of certain substrates, and searches for candidate mediators that could replace Hsp70 in this support role for CHIP are currently underway in our laboratory.

A novel protective role of CHIP against oxidative stress. The protective function of CHIP has been linked to several neurodegenerative diseases,[8] as revealed by the targeting of misfolded proteins such as expanded polyglutamine proteins, CFTR, and tau for proteasomal degradation.[3,40,41] In the present study we propose a novel protective mechanism of CHIP against oxidative stress-induced cell death, in which CHIP regulates EndoG in an ubiquitin proteasome-dependent manner, and the abundance of EndoG determines the sensitivity of cells to oxidative stress. Interestingly, in normal conditions EndoG levels are pre-regulated by CHIP prior to oxidative stress. High levels of EndoG in CHIP-depleted cells resulted in increased caspase-independent cell death upon oxidative stress, whereas low levels of EndoG as a result of CHIP overexpression reduced the incidence of cell death upon oxidative stress in cancer cells and cortical neurons. Moreover, high levels of EndoG concurrent with low levels of CHIP correlated with a higher incidence of cell death upon oxidative stress in the brains of old mice compared with those of young mice. Our data therefore expand our understanding of the protective function of CHIP.

Implications for cancer and aging. Our findings have important implications for cancers and aging. We show that CHIP expression resulted in downregulation of EndoG and reduced cell death upon oxidative stress in breast cancer cells. This is consistent with previous reports that EndoG sensitizes noninvasive breast cancer cells to apoptosis,[20] and that the sensitivity of prostate cancer cells to chemotherapeutic drugs depends on EndoG expression.[21] Maintaining relatively high levels of EndoG in cancer cells may be an effective strategy for accelerated apoptosis in response to anticancer drugs. Our data suggest that high levels of EndoG may be achieved by lowering the expression of CHIP in cancer cells. However, CHIP levels have been negatively correlated with the malignancy of breast tumor tissues, and tumor growth was significantly inhibited by CHIP expression in breast cancer.[24] Further investigation into the molecular networks regulated by CHIP in various types of cancers and the feasibility of inducing EndoG-dependent cell death with different kinds of anticancer drugs by manipulating CHIP levels is required.

The association of CHIP with aging and oxidative stress has been previously demonstrated. CHIP knockout mice exhibit a reduced life span associated with accelerated aging and increased oxidative stress,[9] and CHIP silencing induces premature senescence accompanied by elevated levels of oxidized proteins.[42] In the present study, CHIP depletion led to the enhancement of oxidative stress-induced cell death, and conversely CHIP overexpression resulted in reduced levels of EndoG and cell death upon oxidative stress in primary rat cortical neurons. Moreover, the low levels of CHIP concurrent with high levels of EndoG in the brains of old mice seem to be correlated with a high incidence of cell death on oxidative stress. It has been shown that the reduced levels of CHIP in old mice are regulated at a post-transcriptional level rather than at transcription,[25] and that CHIP is regulated by Ube2W and Ataxin-3 upon completion of substrate ubiquitination,[43] but it remains unknown why and how CHIP levels are maintained at a low level in aged mice. It is likely that an adequate amount of intracellular CHIP would overcome the age-dependent susceptibility to oxidative stress-induced cell death. Elevation or maintenance of CHIP protein could be targeted as a potential therapeutic strategy against oxidative stress-induced cell death associated with aging.

Materials and Methods

Cell culture and transfection. Human cervical cancer HeLa cells were cultured in minimum essential medium, and human embryonic kidney 293 and 293T cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (WelGENE, Seoul, Korea). Transfection was performed using PEI (Sigma, St. Louis, MO, USA), Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), and X-tremeGENE HP DNA Transfection Reagent (Roche, Indianapolis, IN, USA) according to the manufacturer's instructions.

Primary cultures of rat cortical neurons were prepared from embryonic day 15 rats. Cells were microdissected from the brains, and subjected to trypsin digestion and mechanical trituration. The cells were harvested and resuspended in Neurobasal medium (GIBCO, Carlsbad, CA, USA) containing B27, 50 mg/ml

gentamicin, and 2 mM glutamax. The neurons were transfected using the Nucleofector Kit (Lonza, Ratingen, Germany) according to the manufacturer's instructions. The cells were plated in 24-well coverslips coated with poly-L-lysine (0.1 mg/ml, Sigma) and lamine (5 mg/ml, Sigma) at a density of 1×10^6 cells/cm^2. After 48 h transfection the cells were treated with 100 μM H$_2$O$_2$ and 100 μM Z-VAD-FMK for 12 h. For H$_2$O$_2$ treatment the medium was replaced with B27 minus AO (GIBCO).

Immunoprecipitation and immunostaining. Anti-CHIP (Youngin Frontier, Seoul, Korea), anti-actin (Bethyl, Montgomery, TX, USA), anti-green fluorescent protein (GFP; Millipore), anti-EndoG (Pro-sci, Poway, CA, USA), anti-AIF (BD Biosciences, Franklin Lakes, NJ, USA), anti-Myc (Upstate, Billerica, MA, USA), anti-HA (Covance, Emeryville, CA, USA), anti-Hsp70 (Santa Cruz Biotechnology, Inc. Dallas, TX, USA), anti-tubulin (Millipore), and anti-Ubiquitin (Chemicon, Billerica, MA, USA) antibodies were used for immunoblotting or co-immunoprecipitation (co-IP). Co-IP experiments were performed as described below unless otherwise noted. Cells were transfected with the indicated plasmids and lysed in lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 10% glycerol, protease inhibitor cocktail). Cell extracts were mixed with the appropriate antibodies overnight at 4 °C. Immunocomplexes were recovered by incubation with protein-A sepharose (Sigma), and the resin was washed three times with washing buffer (20 mM Tris pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.1% Triton X100, protease inhibitor cocktail). Samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and analyzed by immunoblotting.

For general immunostaining, cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min and permeabilized using 0.2% Triton X-100/PBS for 15 min. Cells were blocked for 1 min in 5% goat serum, and incubated as required with primary antibody (anti-EndoG 1:400, anti-CHIP 1:2000, anti-Hsp70 1:800) for 2 h or overnight in 5% goat serum, followed by incubation with rabbit alexa 546-conjugated or mouse alexa 588-conjugated secondary antibody (Invitrogen). Cells were counterstained with DAPI, mounted (Vector Laboratories, Inc., Burlingame, CA, USA), and visualized using a LSM 510 Meta confocal microscope (Carl Zeiss Inc., Germany).

RNA interference. HeLa cells were transfected with 500 pmol CHIP-specific siRNA (Dhamacon, Chicago, IL, USA) or a scrambled RNA (Genolution, Seoul, Korea) with X-tremeGENE HP DNA Transfection Reagent (Roche). After 24 h incubation the cells were trypsinized and seeded in 24-well plates at 4×10^4 cells/cm^2. After incubation for 24 h the cells were washed with PBS and fixed for terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. Primary rat cortical neurons were transfected with 100 pmol of scRNA or rat siCHIP using the Nucleofector Kit (Lonza). The cells were plated in 24-well coverslips coated with poly-L-lysine (0.1 mg/ml, Sigma) and lamine (5 mg/ml, Sigma) at a density of 1×10^6 cells/cm^2. After 48 h, the neurons were treated with 100 μM H$_2$O$_2$ and 100 μM Z-VAD-FMK for 12 h, and then the coverslips were washed twice with PBS and fixed in 4% paraformaldehyde for TUNEL assay. The sequences of the siRNAs used were as follows: CHIP-specific, 5′-CGCUGG UGGCCGUGUAUUA-3′ (human), 5′-GGAGCAGCGACUCAACUUUUT-3′ (rat); EndoG-specific, 5′-AAGAGCCGCGAGUCGUACGUG-3′ (human).

In vivo ubiquitination assay and measurement of half-life of EndoG. For the in vivo ubiquitination assay 293T cells were transfected as indicated. At 24 h after transfection the cells were treated with 20 μM MG132 for 6 h and lysed in lysis buffer. Ubiquitination was detected by co-IP with anti-GFP antibody and immunoblot analysis with anti-GFP, anti-HA or anti-Ub.

For measurement of the half-life of EndoG 293T cells were co-transfected with empty vector, HA-CHIP or HA-CHIP K30A, and GFP-EndoG WT or GFP-EndoG MLS. At 24 h after transfection the cells were treated with 200 μg/ml cyclohexamide for 0, 4, 8, and 12 h, and harvested at the indicated time points. Cells were lysed with lysis buffers and each sample was subjected to immunoblot analysis with anti-GFP, anti-HA, and anti-actin antibodies.

Reverse transcription-polymerase chain reaction analysis. Total RNA was isolated using easy-spinTM Total RNA Extraction Kit (iNtRON Biotechnology Inc. Seoul, Korea) according to the manufacturer's instructions. cDNA synthesis was carried out using M-MuLV reverse transcriptase (RT-&GO Mastermix, MP Biomedicals, Santa Ana, CA, USA). RT-PCR was performed using a Maxime PCR PreMix kit (iNtRON Biotechnology, Inc.) following the

manufacturer's instructions. PCR products were separated on a 1.8% agarose gel. RT-PCR primers for EndoG were: sense 5′-CGACACGTTCTACCTGAGCA-3′, antisense 5′-AGGATTTCCCATCAGCCTCT-3′.

TUNEL assay. The TUNEL assay was carried out using a TUNEL kit (Promega, Madison, WI). Briefly, cells grown on slides were fixed in 4% paraformaldehyde for 1 h, washed twice with PBS, permeabilized with 0.1% Triton X-100 for 2 min on ice, and washed twice with PBS. Each slide was incubated with solution containing fluorescein-labeled dUTP and terminal deoxynucleotide transferase at 37 °C for 1 h. Following completion of the TUNEL procedure, samples were incubated with DAPI (1 μg/ml) for 10 min and examined under a confocal microscope to count fluorescence-positive and -negative nuclei.

Animals and H$_2$O$_2$ injection. All experiments were approved by and carried out in accordance with the regulations of the Kyung hee University Animal Care and Use Committee (KUACUC). C57BL/6J mice were used for these experiments. All mice were housed at 4–5 animals per cage under controlled conditions of temperature (23.0 $\pm$ 1 °C) and light (12 h light/12 h dark cycles), and food and water were provided ad libitum. Ten-week-old and thirty-week-old mice were microinjected with H$_2$O$_2$ (400 mM in 2 μl saline) or saline only into contralateral sides of the brain aiming at the dentate gyrus (coordinates AP $-$1.8 mm, ML $\pm$ 1.5 mm with respect to Bregma, DV $-$2.15 mm from dura) using a picospritzer III (Parker, Fairfield, NJ, USA). After 3 days the animals were killed for preparation of brain slices.

Preparation of brain slices for staining. Animals were perfused intracardially with 0.1% PBS pH 7.4 followed by 4% paraformaldehyde in PBS. Brains were removed, post fixed in 4% paraformaldehyde in PBS, and stored in 30% sucrose in PBS at 4 °C. Coronal sections through the whole brain were cut at a 30-μm thickness and stored in 30% glycerol, 30% ethylene glycol, 30% PBS at 4 °C until use. For TUNEL staining, brain slices were collected for every fourth section and mounted on gelatin-coated glass slides. The brain sections were analyzed by TUNEL assay (Roche) or stained with cresyl violet for measurement of neuronal loss.

Data analysis. Every fourth section of each brain (from bregma $-$1.84 to $-$2.20) was sampled to measure the damaged area in the dentate gyrus due to H$_2$O$_2$-induced cell death. Areas were measured using the Image J program (freeware from the National Institutes of Health; http://rsb.info.nih.gov/ij/) and the extent of damage was estimated as percent damaged area (Area (%) = 100 $\times$ damaged area in the dentate gyrus/total area of the dentate gyrus). All values were given as mean $\pm$ standard error of the mean (S.E.M.); error bars in figures also represent S.E.M. Data were compared using an unpaired t-test with Prism 4 (Graphpad Software, La Jolla, CA, USA).

Conflict of Interest
The authors declare no conflict of interest.

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