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Novel Oxidative Stress-responsive Gene *ERS25* Functions as a Regulator of the Heat-shock and Cell Death Response*

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Members of the yeast p24 family, including Emp24p and Erv25p, exist as heteromeric complexes that have been proposed to cycle between the endoplasmic reticulum (ER) and Golgi compartments. The specific functions and sites of action of p24 proteins are still unknown. Here we identified a human homolog of the yeast p24 family of proteins, named ERS25 (endoplasmic reticulum stress-response protein 25), and investigated its role in stress response. ERS25 is predicted to have an ER localization signal peptide, a GOLD (Golgi dynamics) domain, which is found in several eukaryotic Golgi and lipid-trafficking proteins, a coiled-coil region, and a transmembrane domain. We demonstrate that ERS25 is localized to the ER and is induced by ER-specific stress, heat shock, and oxidative stress. The selective induction of ERS25 by brefeldin A, but not tunicamycin, implicates the involvement of ERS25 in protein trafficking between the ER and the Golgi. Small interfering RNA-mediated inhibition of ERS25 results in a significant decrease in apoptosis as well as a reduction of reactive oxygen species induced by oxidative stress. Moreover, ERS25 depletion results in a significant increase in the levels of the ER chaperone HSP70 in response to heat-shock stress through increased levels of HSF-1. We also found that inhibition of ERS25 induction in response to heat shock enhanced the binding of HSP70 to Apaf-1, which is likely to interfere in stress-mediated apoptosis. Together, the data presented here demonstrate that ERS25 may play a critical role in regulation of heat-shock response and apoptosis.

The family of p24 proteins is type I membrane proteins with an endoplasmic reticulum (ER)[3] signal peptide and one transmembrane domain. p24 proteins have mainly been studied in yeast and are known to have a GOLD (Golgi dynamics) domain, which is found in several eukaryotic Golgi and lipid-trafficking proteins and predicted to mediate diverse protein-protein interactions (1). In yeast, p24 proteins are synthesized in the ER and packaged into vesicles that bud off from the ER in a GTP-dependent process (2, 3). p24 proteins have been shown to be critical components of the coated vesicles that are involved in the transportation of cargo molecules from the ER to the Golgi complex (4, 5) through the formation of hetero-oligomeric complexes and are believed to function as receptors for specific secretory cargo (1). Two of the p24 family proteins, Emp24p and Erv25p, are localized mainly in the ER in yeast (6, 7), whereas in mammalian cells, the known p24 proteins are localized to the Golgi apparatus and the tubulovesicular compartment on the *cis*-side of the Golgi stack (8–10) with some family members shown to partially reside in the ER (10). It has also been hypothesized that p24 proteins have a role in quality control to prevent misfolded proteins from being loaded into vesicles from the ER (11, 12).

A variety of stress stimuli, including heat shock, UV light, ionizing radiation, and oxidative stress, cause cells to accumulate a highly conserved set of molecules called heat-shock proteins (HSPs) (13, 14). Heat-shock and reactive oxygen species can both result in the accumulation of damaged and misfolded proteins within the cytosol, which is termed the unfolding protein response. A similar, and sometimes overlapping, stress response, known as ER stress, results from the buildup of damaged and misfolded proteins within the ER, resulting in an overload the ER-folding machinery (15–18). The various HSPs, including HSP70, act to bind these proteins and facilitate their repair or degradation within the cytosol. HSPs are highly conserved from prokaryotes to eukaryotes, and most cells induce HSPs of ~25, 70, 90, and 110 kDa (19–21). Among these proteins, the 70-kDa HSP (HSP70) is the most highly induced and conserved from *Escherichia coli* to humans (22), with 95% amino acid sequence homology between human and mouse. Although the basal expression of HSP70 is very low, the induction upon heat shock is rapid and important for survival as mice deficient in HSP70 show genomic instability and enhanced radiosensitivity (22). In addition, down-regulation or disruption of HSP70 expression results in apoptosis (23, 24), whereas cells overexpressing HSPs are protected from hyperthermia (25–28). Several observations that the level of thermotolerance acquired by a cell is quantitatively related to the absolute levels of HSPs, particularly HSP70, suggest that HSPs play a protective role in cell survival (19, 29, 30). Recently a direct link between HSP70 and apoptosis was made when it was discov-

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[3] The abbreviations used are: ER, endoplasmic reticulum; ROS, reactive oxygen species; HSP, heat-shock protein; PBS, phosphate-buffered saline; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; DCF-FA, 2–7-dichlorofluorescein diacetate; shRNA, short hairpin RNA; shERS25, short hairpin ERS25.

ered that HSP70 inhibits the activation of caspase 9 by the Apaf-1 (apoptotic protease-activating factor) apoptosome (31, 32). Thus, a clear role exists for HSP70 in cell survival upon heat-shock stress.

In this study, we present the first cellular characterization of the first ER-localized human homolog of the p24 family of proteins and identify *ERS25* as a stress response gene. Human ERS25 encodes for a polypeptide of 227 amino acids with a molecular mass of 25 kDa, which belongs to the Emp24/gp25L/p24 family of proteins with molecular mass between 23 and 27 kDa. We show that this protein localizes to the ER membrane and is induced by several forms of stress that induce accumulation of unfolded and misfolded proteins. Ablation of ERS25 results in decreased sensitivity to oxidative stress and an increase in HSP70 expression upon heat shock as well as enhanced binding between induced HSP70 and Apaf-1. These results demonstrate that ERS25 is a novel protein of the p24 family of proteins involved in ER stress response and is an important component of heat-shock regulation, representing a novel link between p24 family proteins and ER-stress mediated cell death pathway.

EXPERIMENTAL PROCEDURES

Cell Culture, Transfection, and Heat-shock Treatment—NIH3T3 and U2OS cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Invitrogen) and 100 units/ml penicillin and streptomycin at 37 °C, 5% CO_2. NIH3T3 cells were transfected with a pBabe-U6 plasmid using Lipofectamine 2000 (Invitrogen), stable cells were selected for, and the clones were maintained in 1 μg/ml puromycin. For heat-shock treatment or chemical treatment, cells were plated at a concentration of 1×10^6 cells in a p100 plate, and 24 h later, the cells were placed at 42 °C, 5% CO_2 as indicated. Chemicals used for inducing stress were purchased from Sigma, including tunicamycin, brefeldin A, and hydrogen peroxide (H_2O_2).

shRNA Experiments—Short hairpin RNA oligonucleotides against mouse ERS25 were ordered from Dharmacon, and the following sequence was determined to be the most effective and used in all experiments: 5′-AAGGA GCAGG ATTAT CAG-3′. NIH3T3 cells were transfected with either pBabe-U6 plasmid expressing shRNA targeting ERS25 or empty vector as a control.

Protein Analysis—Cells were washed with ice-cold PBS and lysed in lysis buffer (20 mM Tris (pH 7.4), 5 mM EDTA, 10 mM $Na_4P_2O_7$, 100 mM NaF, 2 mM Na_3VO_4, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1× Roche Applied Science protease inhibitor mixture). An equal amount of total cellular proteins per sample was run on a SDS-PAGE and transferred to a nitrocellulose membrane (Invitrogen). Antibodies used for Western blotting were: anti-Apaf-1 (BD Biosciences), anti-β-actin (AC-15, Sigma), anti-calnexin (Santa Cruz Biotechnology), anti-ERS25 (Imgenex), anti-HSP70 (StressGen), anti-poly(ADP-ribose) polymerase-2 (Calbiochem), and anti-α-tubulin (Sigma). For immunoprecipitation, CHAPS buffer was used to lyse the cells and protein A-agarose beads (Sigma) were used for immobilization.

Northern Blot Analysis—Total RNA was extracted using the PureLink Micro-to-Midi total RNA purification system with homogenizers (Invitrogen) according to the manufacturer's protocol. Samples were quantified and denatured, and equal amounts were electrophoresed through 1% agarose gel by the formaldehyde denaturation method. RNA was transferred to a nylon membrane (Bio-Rad), UV-cross-linked (Stratagene), and then baked at 80 °C for 1 h. Hybridization was performed with ^{32}P-labeled probes prepared by the random prime DNA labeling method (Invitrogen).

Subcellular Fractionation—Cells were washed twice with ice-cold PBS, hypotonic buffer (20 mM Hepes (pH 7.9), 10 mM KCl, 0.1 mM Na_3VO_4, 1 mM EDTA, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 20 mM NaF, 1 mM dithiothreitol, and protease inhibitor mixture) added directly to the plates and incubated on ice for 10 min. Cell lysates were collected, and 0.2% Nonidet P-40 was added for 1 min. A 1-ml syringe with 25-gauge needle was used for disrupting cells. Cytosolic proteins were centrifuged at 13,000 rpm for 1 min. The pellet was the washed with hypotonic buffer, and high salt buffer (hypotonic buffer supplemented with 20% glycerol and 420 mM NaCl) was added to extract nuclear proteins by rocking overnight at 4 °C. The resulting supernatant was taken for nuclear extract, and the pellet was dissolved in lysis buffer to collect the membrane fraction.

Immunostaining—Cells were plated on poly-L-lysine-coated chamber slides (BD Biosciences) and attached for 24 h before heat-shock treatment. Cells were fixed in 3.7% formaldehyde in PBS for 20 min before permeabilization in 0.2% Triton X-100, blocked with 3% bovine serum albumin in PBS, 0.2% Triton X-100, and incubated overnight at 4 °C with primary antibodies (1:500). After washing three times with PBS, 0.2% Triton X-100, the cells were incubated with secondary antibodies (1:500) for 1.5 h at room temperature. Cells were washed three times with PBS, 0.2% Triton X-100 and stained for 20 min with the nuclear dye TO-PRO3 iodide (1:2000, Invitrogen). Slides were sealed with coverslips, and images were collected on a Leica TCS4D confocal laser-scanning microscope. Images were processed using Adobe PhotoShop software. Antibodies used for immunostaining were: anti-ERS25/HNLF (Imgenex) and calnexin (Santa Cruz Biotechnology).

Luciferase Assay—A reporter plasmid containing the putative *HSP70* recognition sequence in the promoter was transfected into NIH3T3 cells. The HSP70-pr-Luciferase plasmid was a generous gift from Dr. Morimoto (Northwestern University). pRL-TK (*Renilla* plasmid) was also co-transfected for normalizing the luciferase signal. Cells were harvested 48 h after transfection, and the assay was performed using the Dual-Luciferase reporter assay system (Promega), after a 4-h incubation at 37 or 42 °C.

Cell Death Assays—Cell death was measured using a cell death detection ELISA (Roche Applied Science) and trypan blue exclusion. Cells used for the ELISA were analyzed according to the manufacturer's protocol to measure the amount of DNA fragmentation. Trypan blue exclusion was performed on cells harvested and resuspended in medium and mixed 1:1 with 0.4% trypan blue. The percentage of cell survival was deter-

mined from the number of cells that stain blue *versus* the total cell count.

Propidium Iodide Staining and Flow Cytometry—Cells were harvested and fixed with 70% ethanol while gently vortexing. Cells were stored at 4 °C up to 1 week prior to analysis. Prior to analysis, the cells were washed with PBS and incubated in PBS containing 500 μg of RNase A for 30 min at 37 °C followed by a 15-min room temperature incubation with 25 μg of propidium iodide. Cells were analyzed on a FACSCalibur (BD Biosciences), and the data were analyzed using FlowJo 6.3.4 (Tree Star Inc.).

Detection of Intracellular ROS—Cells were treated with various concentrations of H_2O_2 for 10 min, and then 10 μg/ml 2–7-dichlorofluorescein diacetate (DCF-DA, Fluka) was added for 30 min at 37 °C. The cells were harvested by trypsinization and resuspended in PBS. Intracellular reactive oxygen species oxidize DCF-DA, resulting in an increase in fluorescence as measured by flow cytometry as described above.

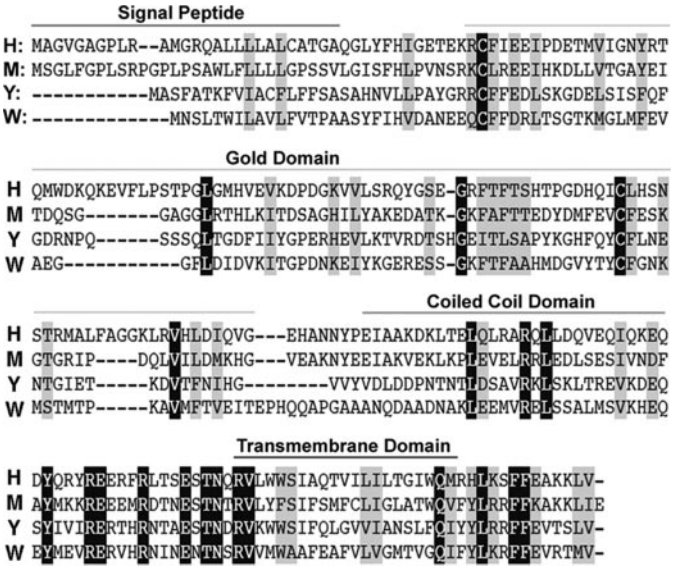

FIGURE 1. **Sequence alignment of Emp24 family proteins.** *Homo sapiens* (*H*) human ERS25 (NP_872353.2), *Mus musculus* (*M*) mouse Tmed10 (NP_081051.1), *Saccharomyces cerevisiae* (*Y*) yeast Emp24 (NP_011315.1), and *Caenorhabditis elegans* (*W*) worm sel-9 (NP_505145.1). Amino acids highlighted in *black* are 100% conserved, and those highlighted in *gray* are conservative substitutions. The domains conserved within the Emp24 family are labeled by *dashes*.

RESULTS

Identification of ERS25—We identified a novel gene termed *ERS25* as an oxidative stress-response gene by microarray analysis of cDNA expression following H_2O_2 treatment in U2OS cells. Human *ERS25* encodes for a polypeptide of 227 amino acids and belongs to the Emp24/gp25L/p24 family of proteins. ERS25 is conserved across species including human, mouse, yeast, and worm (Fig. 1*A*). Domain analysis of the ERS25 sequence indicated that ERS25 contains an N-terminal signal peptide for targeting to the ER, a large GOLD (Golgi dynamics) domain, found in Golgi and lipid-trafficking proteins (1), a coiled coil domain, and one transmembrane region (Fig. 1).

To characterize the function of this novel p24 family protein, ERS25, we first studied its subcellular localization by cellular fractionation and immunocytochemistry. Since topology predictions indicate an ER signal peptide and transmembrane domain in ERS25 (Fig. 1), we expected ERS25 to be localized in the membrane of compartment of the secretory pathway. Endogenous ERS25 was observed by confocal microscopy in NIH3T3 cells stained with antibodies against ERS25 and calnexin, an ER marker, with TO-PRO3 staining of the nucleus. ERS25 clearly co-localized with calnexin in the ER (Fig. 2*A*). Cellular fractionation shows that ERS25 was located in the membrane fraction, which includes the plasma membrane, ER, and Golgi apparatus (Fig. 2*B*). ERS25 was not detected in either the nuclear or the cytosolic fractions (Fig. 2*B*).

To further investigate whether ERS25 was involved in ER or oxidative stress responses, we exposed NIH3T3 cells to ER pertubators such as brefeldin A and tunicamycin, oxidative stress (1 mM H_2O_2), and heat shock (42 °C). These stresses are known to result in the accumulation of unfolded and unsecreted proteins within the cytosol and ER and cause a stress response depending on their severity. ERS25 was significantly induced by treatment with brefeldin A, hydrogen peroxide, and heat shock (Fig. 2*C*),

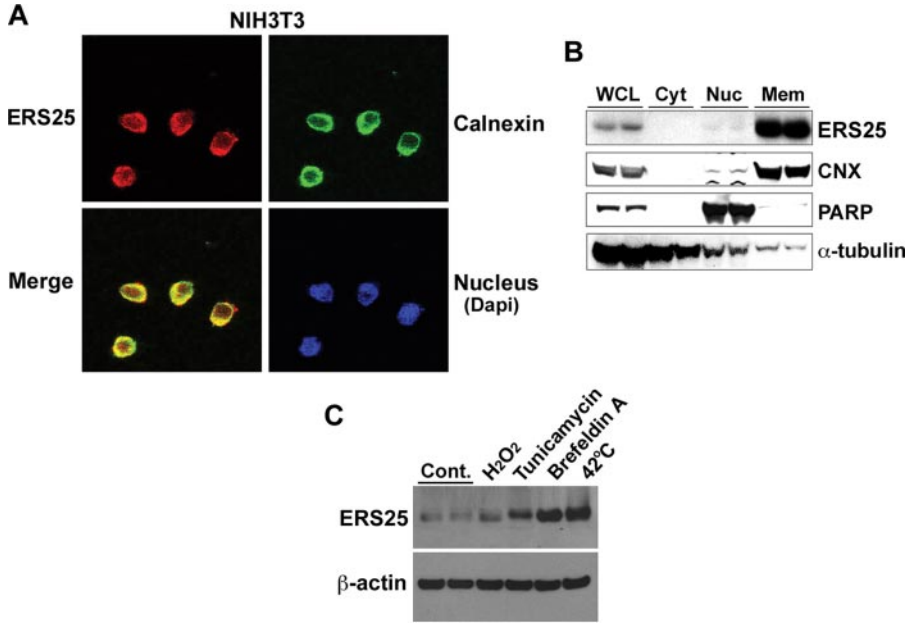

FIGURE 2. **ERS25 localizes to the ER and is induced by various stressors.** *A*, NIH3T3 cells were stained with antibodies against ERS25 and calnexin, and the nucleus was stained with TO-PRO3 iodide. *Dapi*, 4′,6-diamidino-2-phenylindole. *B*, Western blot analysis of NIH3T3 cell fractionation. ERS25 was identified in the membrane fraction using antibodies against calnexin (*CNX*), poly(ADP-ribose) polymerase-2 (*PARP*), and α-tubulin, as markers for the membrane (*Mem*), nuclear (*Nuc*), and cytosolic (*Cyt*) fractions, respectively. *WCL*, whole cell lysates. *C*, NIH3T3 cells were stressed using tunicamycin, brefeldin A, 1 mM H_2O_2, and 8 h of heat shock at 42 °C. Western blot analysis was performed with antibodies against ERS25 and β-actin as a loading control (*Cont.*).

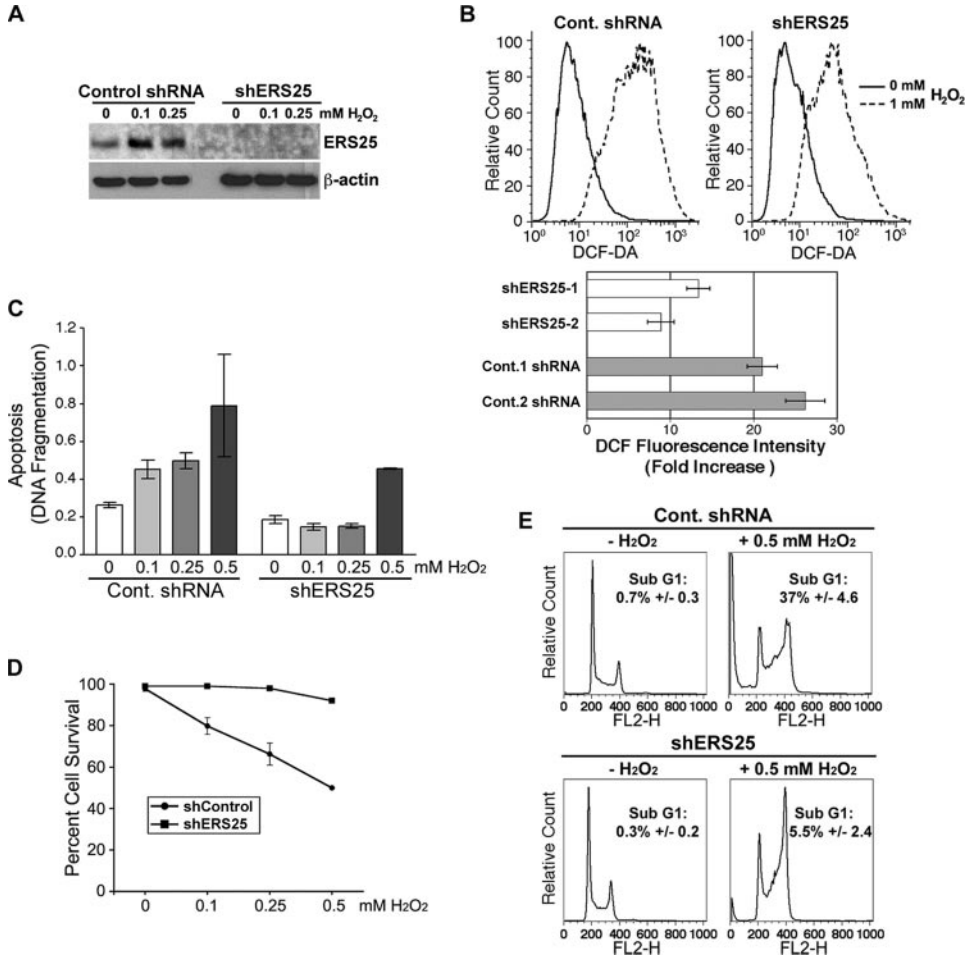

FIGURE 3. **ERS25 sensitizes cells to oxidative damage and cell death.** NIH3T3 cells were stably transfected with either empty pBabe-shRNA vector (control) or vector containing shRNA targeting ERS25. Cells were treated with various concentrations of H_2O_2 for 16–18 h. *A*, Western blot analysis of H_2O_2-treated cells using antibodies against ERS25 and β-actin as a loading control. *B*, reactive oxygen species were measured by DCF-DA fluorescence as described under "Experimental Procedures." Various cell death assays were performed on H_2O_2-treated cells as described under "Experimental Procedures." *Cont.*, control. *C*, apoptosis was quantified by a cell death enzyme-linked immunosorbent assay (Roche Applied Science) measuring DNA fragmentation. *D*, the percentage of cell survival was quantified by trypan blue exclusion. *E*, apoptosis was visualized by fluorescence-activated cell sorter analysis of propidium iodide-stained cells. The percentage of sub-G_1 population and *error bars* reflect the mean ± S.D. from duplicate experiments.

whereas remaining at basal levels upon treatment with tunicamycin, exposure to hyperosmotic stress (data not shown), or serum starvation (data not shown), suggesting that ERS25 is involved in the ER stress response regulation.

Inhibition of ERS25 Impairs by Oxidative Stress-mediated Apoptosis—To investigate the role of ERS25 in mediating oxidative stress-mediated apoptosis, we used the pBabe-U6-shRNA retroviral vector system to create ERS25 knockdown (shERS25) and control (empty vector) constructs and evaluated the effect of inhibiting endogenous ERS25 expression on apoptosis induced by H_2O_2. Transfection of ERS25 shRNA resulted in the suppression of ERS25 induction by H_2O_2 treatment, whereas the control had no effect on induced ERS25 levels in NIH3T3 cells (Fig. 3*A*). We generated several stable clones and used these clones to make sure our results were not due to clonal variation. To understand the role of ERS25 in oxidative stress, we treated the cells with various concentrations of H_2O_2 and measured the level of reactive oxygen species (ROS) by monitoring the oxidation of the dye DCF-DA by fluorescence-activated cell sorter analysis. The ERS25 knockdown cells generated less ROS upon treatment with H_2O_2 as seen by the decreased right-shift in DCF-DA signal, as compared with control-transfected cells (Fig. 3*B*). Furthermore, the ERS25 knockdown cells clearly showed a significant reduction in the percentage of apoptotic cells as compared with control cells after exposure to H_2O_2 (Fig. 3, *C–E*), as demonstrated by three different cell death assays. Thus, depletion of ERS25 expression by shERS25 was effective in inhibiting ROS levels and apoptosis induced by oxidative stress after exposure to H_2O_2.

Inhibition of ERS25 Expression Enhances HSP70 Induction by Heat-shock Response—Having demonstrated that ERS25 is localized in the ER, suggesting that it functions in ER machinery and observing that it is up-regulated by heat shock similarly to HSPs, we were then interested in delineating how ERS25 is involved in the pathway of heat shock. We first looked for changes in expression of HSPs in response to heat shock with or without ERS25 depletion. ERS25 knockdown or control cells were incubated at 42 °C for various times. As shown in Fig. 4, as early as 4 h after heat-shock, ERS25 levels began to be increased and, as expected, the heat-shock protein 70 (HSP70) was significantly induced in response to heat shock at 42 °C. Surprisingly, we found that the knockdown of ERS25 expression/induction in response to heat shock enhanced HSP70 protein expression in multiple clones (Fig. 4*B*), but there was no effect on other HSPs (data not shown). We also determined whether the increase in the HSP70 protein resulted from an increase of HSP70 mRNA. We found that the knockdown of ERS25 also significantly enhanced the levels of HSP70 mRNA as early as 2 h of heat shock, suggesting that the increase of HSP70 induction was likely transcriptional and that ERS25 functions as an upstream regulator of *HSP70* gene transcription in response to heat shock.

Therefore, we evaluated whether ERS25 could modulate *HSP70* gene transcription using a luciferase reporter assay of the *HSP70* promoter. NIH3T3 cells transfected by control (empty vector) or shERS25 were transfected with HSP70-luciferase plasmid as well as pRL-TK *Renilla* luciferase, and then empty pGL3 luciferase plasmid was used as a control. 24 h after transfection, cells were incubated at 42 °C for 4 h to induce the maximum amount of HSP70. The empty pBabe-vector-trans-

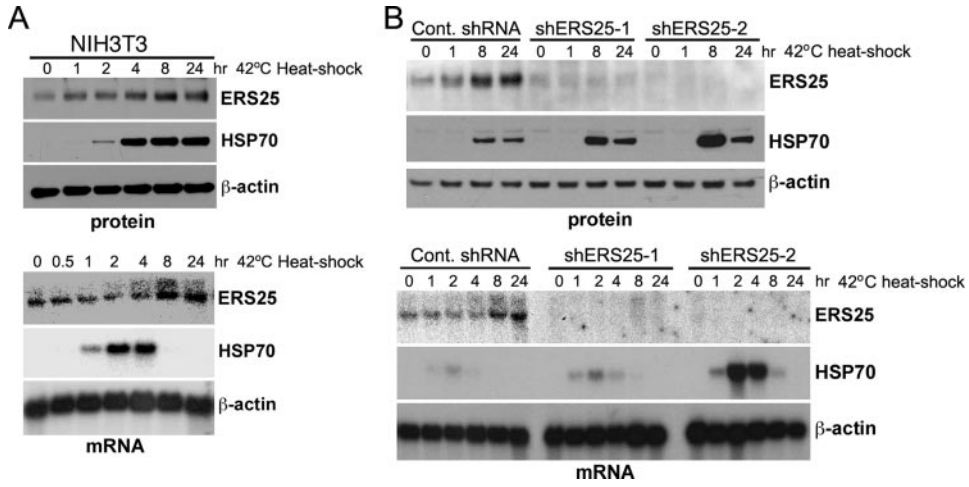

FIGURE 4. **Inhibition of ERS25 enhances HSP70 induction upon heat shock.** *A*, NIH3T3 cells were exposed to heat shock (42 °C) for the indicated times and then harvested for analysis by Western (*upper panel*) and Northern (*lower panel*) analysis. The levels of ERS25 and HSP70 were measured with β-actin and 36B4 as protein and mRNA controls, respectively. *B*, NIH3T3 cells were stably transfected with either empty pBabe shRNA vector (control (*Cont.*)) or vector containing shRNA targeting ERS25. Two clones were analyzed for protein and mRNA as described in *A*.

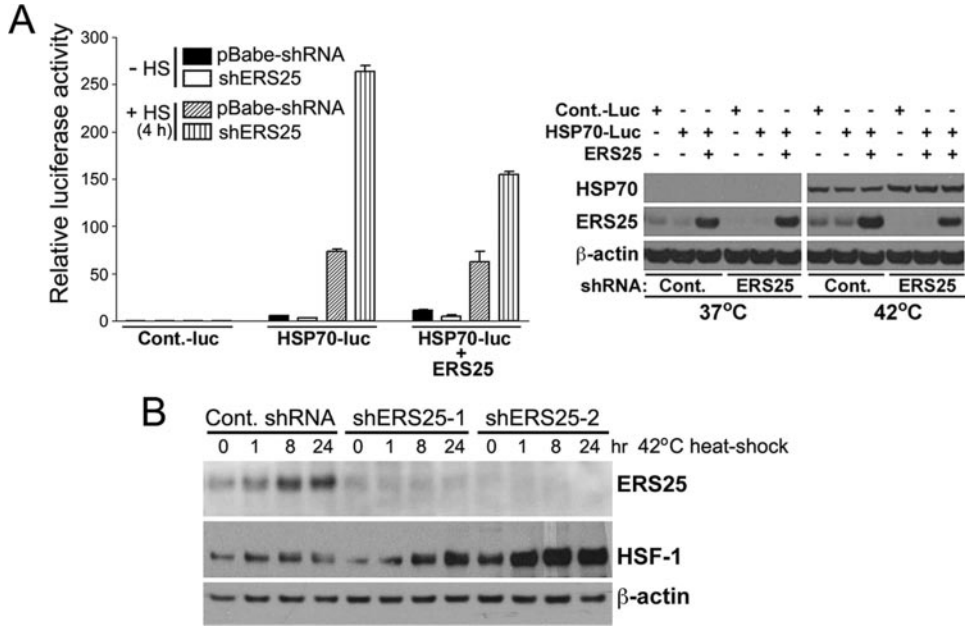

FIGURE 5. **ERS25 knockdown induces HSF-1 expression and enhances *HSP70* promoter activity.** *A*, luciferase assay reporter gene constructs containing the HSP70 promoter were transfected with the reporter plasmids into NIH3T3 cells expressing the empty pBabe vector or vector expressing shRNA against ERS25. Cells were harvested 48 h after transfection and assayed for luciferase activity. Re-expression of ERS25 upon the transfections was confirmed by Western blot (*right panel*). *HS*, heat shock; *Cont.*, control. *B*, the effect of ERS25 depletion on HSF-1 expression upon heat shock. NIH3T3 cells were transfected with ERS25-shRNA or control shRNA followed by heat shock at 42 °C, and then Western blotting was performed against ERS25, HSF-1, and β-actin.

fected control NIH3T3 cells showed an increase in relative luciferase activity after 4 h of heat shock (Fig. 5*A*). However, ERS25 knockdown cells showed much higher induction in luciferase activity. Moreover, reintroduction of an ERS25-expressing plasmid into the ERS25 knockdown cells decreased induction of HSP70-luciferase activity after heat shock (Fig. 5*A*), indicating that HSP70 up-regulation is likely transcriptional. It is well established that HSF1 transcription factor is a regulator of HSP70 transcription activity in response to heat shock (33–35). We next investigated whether ERS25-mediated modulation of HSP70 transcription is actually due to the expressional change of HSF1. Fig. 5*B* shows that the level of HSF1 protein expression in cells transfected with shERS25 was significantly increased following heat-shock stress, as compared with control-transfected cells. Taken together, these data suggest that ERS25 functions as an effector in heat-shock response by maintaining the levels of HSP70 and HSF1.

ERS25 Inhibition Enhances Apaf-1 Binding to HSP70—It is clear that the cellular-stress response can mediate cellular protection through expression of HSP70, which can interfere with the process of apoptotic cell death (19, 29, 30). Previous reports strongly indicate that the antiapoptotic effect of the principal heat-shock protein, HSP70, is mediated through its direct association with the caspase-recruitment domain (CARD) of Apaf-1 and through inhibition of apoptosome formation (31, 32). To understand the potential mechanism by which ERS25 regulates Hsp70 expression in heat-shock response, we investigated the involvement of ERS25 in HSP70-mediated negative regulation of Apaf-1 apoptosome. We characterized the specificity of the interaction between Apaf-1 and HSP70 *in vivo* with or without ERS25 expression following heat-shock stress by immunoprecipitation. As shown in Fig. 6, ERS25 depletion facilitated increased binding between HSP70 and Apaf-1 apoptosome, whereas no binding between these two proteins was detected without heat-shock stress. The data suggest that ERS25 induction in response to heat-shock stress plays a role in maintaining functional apoptosome by regulating HSP70 levels.

DISCUSSION

We identified *ERS25* as a novel oxidative stress-responsive gene through a cDNA expression array. Human ERS25 encodes for a 227-amino-acid protein with a molecular mass of 25 kDa and belongs to the Emp24/gp25L/p24 family proteins of yeast (Fig. 1). The precise functions of the p24 family of proteins have not been determined, although genetic, molecular, and biochemical analyses suggest that the proteins are possibly

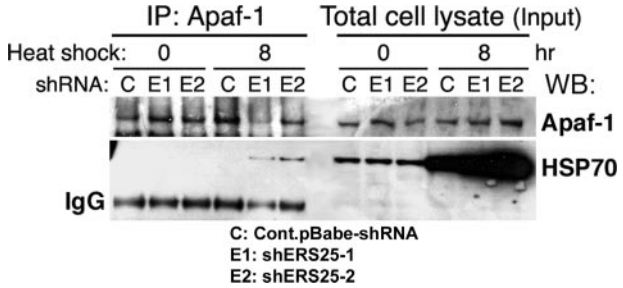

FIGURE 6. **ERS25 reduces HSP70 binding to Apaf-1.** NIH3T3 cells expressing the empty pBabe vector or vector expressing shRNA against ERS25 were exposed to heat shock (42 °C) for 8 h, and cell lysates were used to immunoprecipitate (*IP*) with an Apaf-1 antibody and Western blot (*WB*) for HSP70. *Cont.*, control.

involved in cargo protein selection and packaging, vesicle biogenesis and function, and quality control of protein movement through the secretory system (1, 4, 5). From recent literature, p24 proteins may serve as quality control agents that prevent misfolded or otherwise aberrant proteins from being loaded into vesicles (11, 12, 36–39) and may regulate cell-cell signaling by controlling the amount and timing of protein movement to the surface of the cells. ERS25 contains an N-terminal signal peptide for targeting to the ER, which we have confirmed by immunofluorescence and subcellular fractionation. The ER localization of ERS25 is a unique feature as compared with the other known family members, which are mainly localized to the *cis*-Golgi network (8–10), although human Gmp25 is known to partially localize in the ER (10). We demonstrate that ERS25 is induced by a variety of stress inducers including ER stress, oxidative damage, and heat shock. The common element among all these stress responses is that these stresses are known to cause the accumulation of misfolded proteins in the cytosol and the nucleus. Interestingly, tunicamycin, which causes ER stress by inhibiting glycosylation (40, 41), did not cause an increase in ERS25, but brefeldin A, which causes ER stress by inhibiting protein transport into the Golgi (42), was able to strongly induce ERS25. These data imply that ERS25 may be involved in facilitating protein transport, rather than being involved in general stress response to the accumulation of excess protein in the ER. This is consistent with current data on the Emp24/gp25L/p24 family of proteins that are known to be critical components of the coated vesicles that are involved in the transportation of cargo molecules from the ER to the Golgi (4, 5).

Significantly, shRNA-directed inhibition of ERS25 attenuates oxidative stress-induced ROS and abrogates apoptotic cell death upon H_2O_2 treatment. However, the overexpression of ERS25 alone is insufficient to induce apoptosis (data not shown), and further studies are required to elucidate how ERS25 contributes to ROS generation. The p24 family of proteins has been implicated in the unfolded protein response (11). HSP70 family proteins are known to play important roles in preventing misfolding and aggregation of newly synthesized or unfolded proteins (43–46). We demonstrate that ERS25 is induced by heat shock and abrogation of ERS25 expression in NIH3T3 cells resulted in increased HSP70 expression upon heat shock. Thus, it is predictable that the knockdown of ERS25 levels may cause a compensatory increase of HSP70 levels to cope with the cellular stress and accumulation of unfolded proteins. It has been proposed that HSP70 up-regulation prevents apoptosis at the level of the apoptosome (31, 32). Recent studies have demonstrated that Apaf-1 and HSP70 interaction occurs *in vivo* and in a cell-free system and that HSP70 binds to Apaf-1 and inhibits caspase-9 cleavage, therefore inhibiting cell death (31, 32). Due to the increased expression of HSP70 in ERS25 knockdown cells and the ability of these cells to resist oxidative damage after treatment with H_2O_2, we looked for any alteration in the ability of HSP70 to bind Apaf-1 in the ERS25 knockdown cells. We observed increased levels of binding between HSP70 and Apaf-1 in these cells, which would imply that the ERS25 knockdown cells may be more resistant to apoptosis. This is likely due to the increased expression of HSP70 in the ERS25 knockdown cells. Consistent with the previous reports, we were able to clearly see the increased resistance of ERS25 knockdown cells to apoptosis induced by oxidative damage.

ERS25 is a novel protein, which is located in the ER and is induced by stressors that result in the accumulation of unfolded or misfolded proteins in both the cytosol and the ER. It is probable that ERS25 is induced to increase transport of these proteins to the Golgi. In our characterization of ERS25, we demonstrate that ERS25 is important for the induction of apoptosis upon oxidative damage and for proper modulation of HSP70 expression upon heat shock. The function of ERS25 in the ER remains unclear; however, our data seem to indicate that it plays a role to facilitate protein transport rather than assisting in the clearance of the excess accumulation of protein induced upon stress. It is possible that ERS25 is necessary for the proper processing of other stress response proteins, and therefore, the cell undergoes an aberrant stress response in the absence of ERS25. Further investigations into ERS25 are necessary to elucidate its role in the stress response.

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