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Sep15, a Thioredoxin-like Selenoprotein, Is Involved in the Unfolded Protein Response and Differentially Regulated by Adaptive and Acute ER Stresses[†]

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ABSTRACT: The accumulation of misfolded proteins in the endoplasmic reticulum (ER) results in activation of signaling pathways collectively known as the unfolded protein response (UPR). The UPR promotes adaptation of cells to ER stress by transient inhibition of protein translation and transcriptional up-regulation of genes encoding chaperones, oxidoreductases, and ER-associated degradation components. However, it may also trigger apoptosis in response to persistent ER stress. Recently, a novel selenocysteine-containing oxidoreductase, Sep15, has been reported to reside in the ER lumen. It has been proposed that this oxidoreductase may assist oxidative folding and structural maturation of N-glycosylated proteins targeted by UDP-glucose:glycoprotein glucosyltransferase, a chaperone implicated in quality control in the ER that forms a 1:1 complex with Sep15. To address the role of Sep15 in protein folding, we analyzed changes in Sep15 expression in murine fibroblast NIH3T3 cells in response to tunicamycin, brefeldin A (brefA), thapsigargin, and DTT that lead to accumulation of unfolded proteins within the ER. We show that expression of this protein is transcriptionally up-regulated in response to adaptive UPR caused by tunicamycin and brefA, whereas acute ER stress caused by DTT and thapsigargin leads to rapid and specific degradation of Sep15 by proteasomes. However, Sep15 deficiency did not result in detectable ER stress, consistent with the idea that Sep15 assists in the maturation of a restricted group of N-glycosylated proteins and/or that its function may be compensated by other mechanisms.

The endoplasmic reticulum (ER)[1] provides a suitable oxidative environment for folding and maturation of membrane and secretory proteins. It contains a complex network of molecular chaperones, thiol-disulfide oxidoreductases (enzymes that catalyze formation of disulfide bonds), and quality control machinery required for folding and assembly of newly synthesized polypeptide chains. The folding of N-linked glycoproteins is assisted by an ER chaperone calnexin (CNX), which specifically binds to the monoglucosylated glycan core of glycoproteins (*1*−*3*). CNX forms a functional complex with the ER resident oxidoreductase, ERp57, leading to formation and isomerization of disulfide bonds (*4*−*6*). Eventually, the innermost glucose residue in N-glycans is cleaved by glucosidase II, and glycoproteins are released from CNX. Another component in this system, UDP-glucose:glycoprotein glucosyltransferase (UGGT), functions as the folding sensor, which monitors the folding state of polypeptides released from CNX (*7*). Partially unfolded or improperly folded glycoproteins prematurely released from CNX are reglucosylated (*8*). Addition of glucose allows reassociation of immature proteins with CNX for further disulfide bond formation and folding (*9*−*11*). Finally, correctly folded glycoproteins are released from the CNX cycle. However, extensively misfolded glycoproteins that fail to acquire the native structure are removed from the ER by an ER-associated degradation (ERAD) pathway to avoid their accumulation and ER overload (*12*).

Recently, it has been reported that CNX substrates can be grouped in three classes according to their rates of release from CNX in UGGT-deficient mouse embryonic fibroblasts (*13*). Glycoproteins of the first class did not require UGGT for correct folding, and their folding was accomplished in one binding event. In the second class, glycoproteins depended on UGGT for reassociation with CNX for folding. As a result, these glycoproteins were prematurely released from CNX in cells lacking UGGT. Unexpectedly, CNX release of glycoproteins of the third class was delayed by UGGT deletion, suggesting that UGGT activity and/or associated factors might be required for a structural maturation needed for substrate dissociation from CNX and export from the ER.

It has been proposed that the selenocysteine (Sec)-containing protein Sep15 that forms a 1:1 complex with UGGT could play a role in assessing and refining the disulfide bond content of glycoproteins in this class (*13*, *14*). Sep15 is a thioredoxin-like fold ER-resident protein predicted to function as oxidoreductase (*15*). It lacks an ER retention signal and is kept in the ER due to its tight association with UGGT (*16*).

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[1]Abbreviations: BrefA, brefeldin A; CA1-CA3, Ammon's horn CA1-CA3 regions; CNX, calnexin; DG, dentate gyrus; DTT, dithiotreitol, ER, endoplasmic reticulum; ERAD, ER-associated degradation; RNAi, RNA interference; Sec, selenocysteine; SelP, selenoprotein P; Sep15, 15-kDa selenoprotein; UGGT, UDP-glucose:glycoprotein glucosyltransferase; UPR, unfolded protein response.

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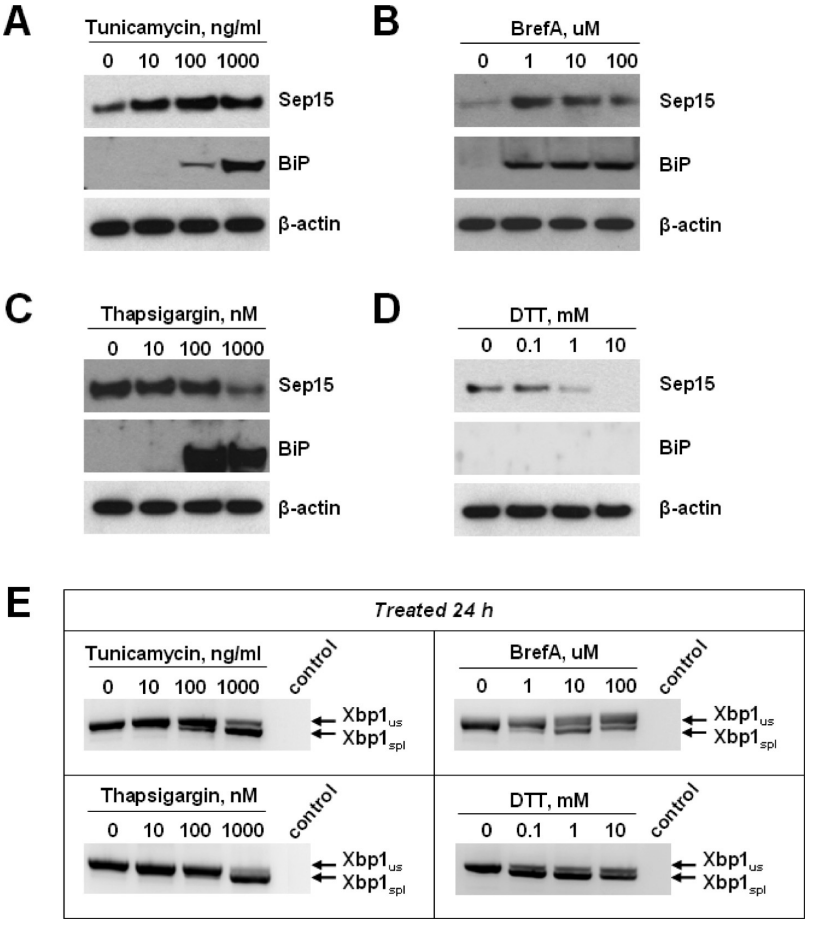

FIGURE 1: ER stress may either enhance or attenuate expression of Sep15. NIH3T3 cells were incubated in the presence of indicated concentrations of tunicamycin (A), brefA (B), thapsigargin (C), or DTT (D) for 24 h. Protein lysates were probed by immunoblot analysis with polyclonal antibodies specific for Sep15 and BiP, or monoclonal β-actin-specific antibody as a loading control. (E) Total RNA was isolated from cells treated with the indicated concentrations of the ER stressors for 24 h. Spliced (*spl*) and unspliced (*us*) *Xbp1* mRNA were detected by RT-PCR. The image was inverted to negative for better clarity. The extent of *Xbp1* mRNA splicing has been quantified and plotted in Figure S1, Supporting Information.

Accumulation of unfolded proteins in the ER or ER stress results in activation of unfolded protein response (UPR) (*17*). UPR is a signaling pathway that leads to inhibition of protein translation and enhanced expression of proteins that facilitate protein folding and help cells to remove misfolded proteins from the ER (*18*, *19*). We hypothesized that ER stress would also regulate expression of Sep15, which may affect protein folding processes (*14*).

Here we show that Sep15 is regulated by ER stress, but this regulation depends on the specific stressor employed. Sep15 expression was increased in response to tunicamycin and brefeldin A (brefA), but rapidly decreased by acute ER stress caused by DTT and thapsigargin. However, Sep15 deficiency per se did not result in detectable ER stress suggesting that Sep15 controls folding of a restricted group of N-glycosylated proteins and that other factors/mechanisms may compensate for Sep15 function.

MATERIALS AND METHODS

Cell Culture. Mouse NIH3T3 cells were cultured in DMEM medium supplemented with 10% FBS, 100 units/mL penicillin, and 100 units/mL streptomycin. ER stress was induced by incubating the cells in the presence of indicated concentrations of tunicamycin, brefA, thapsigargin, and DTT for 24 h. Cells were lysed in CelLytic-M lysis buffer (Sigma, St. Louis, MO). Protein lysates were resolved by SDS−PAGE and analyzed by Western blotting using antibodies specific for Sep15, BiP, and β-actin.

To study degradation of Sep15, NIH3T3 cells were grown in DMEM supplemented with freshly neutralized [^{75}Se]selenious acid (specific activity 1000 Ci/mmol, final concentration in the medium 1 nM) for 24 h. Following metabolic labeling, the medium was replaced and cells were incubated in DMEM containing indicated concentrations of DTT in the presence or absence of 10 μM proteasome inhibitor Z-Leu-Leu-Leu-al (Sigma, St. Louis, MO) for an additional 24 h. Expression of Sep15 and UGGT was assessed by Western blotting. Expression patterns of selenoproteins were analyzed by autoradiography of SDS−PAGE gels using a PhosphorImager.

Xbp1 mRNA Splicing and Real-Time RT-PCR Analyses. Total RNA was extracted from cultured cells by RNAqueous kit (Ambion, Austin, TX) according to the manufacturer's instructions. First strand cDNA was synthesized using the SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA) with random hexamer primers. For analysis of *Xbp1* mRNA splicing RT-PCR was performed with the following primers that flank the *Xbp1* intron: 5′-AAACAGAGTAGCAGCGCAGACTGC-3′ and 5′-TCCTTCTGGGTAGACCTCTGGGAG-3′. For real-time RT-PCR synthesized cDNAs were amplified using Power SYBR Green PCR Master Mix and 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA). The following

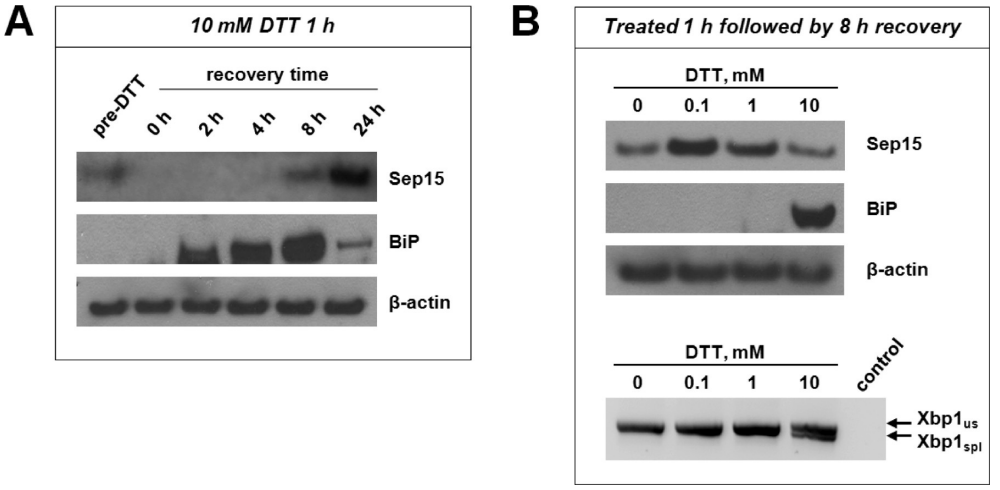

FIGURE 2: Adaptive UPR induced by brief treatment with DTT increases expression of Sep15. (A) NIH3T3 cells were treated with 10 mM DTT for 1 h; the media was replaced with DTT-free media followed by 2−24 h recovery. At the indicated time points protein lysates were prepared and probed by immunoblot analysis with Sep15 and BiP-specific polyclonal, or β-actin-specific monoclonal antibodies. (B) Cells were treated with the indicated concentrations of DTT for 1 h followed by 8 h recovery in DTT-free media, and protein lysates were probed for expression of Sep15, BiP, and β-actin by immunoblot analyses. For analysis of *Xbp1* mRNA splicing, total RNA was isolated and assessed by RT-PCR.

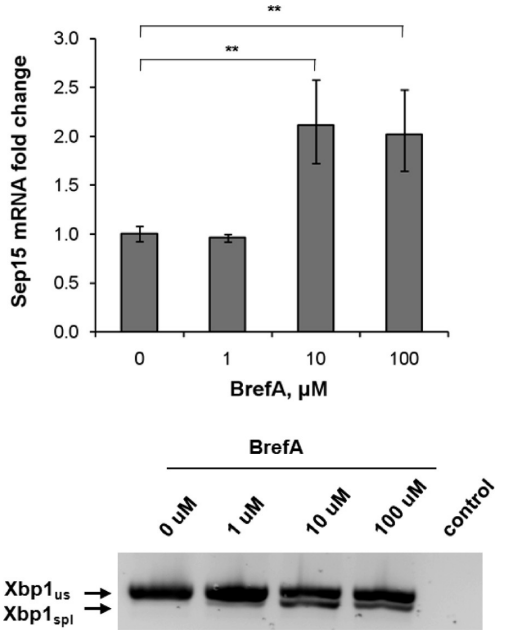

FIGURE 3: Expression of Sep15 is induced by ER stress at the transcriptional level. ER stress was induced in NIH3T3 cells by treatment with indicated concentrations of brefA for 24 h. Total RNA was isolated and the expression of *Sep15* mRNA normalized against β-actin was measured by real-time RT-PCR. Results are represented as means ± SEM of three independent experiments. **, $p < 0.05$. *Xbp1* mRNA splicing was analyzed by RT-PCR. One representative image out of three independent experiments is shown.

primer pairs were used: Sep15: 5′-AAGTCTGCGGATGAAAATTG-3′ and 5′-CCACACTGTCTGTGTTCCAC-3′; β-actin: 5′-CGTGAAAAGATGACCCAGAT-3′ and 5′-CATGAGGTAGTCCGTCAGGT-3′. Each primer pair was designed to span an intron. To validate the real-time RT-PCR assay, Sep15 and β-actin primer sets were tested using a dilution series of cDNA. For both primer sets, PCR efficiency was near 100%. To confirm the amplification of the specific product, melting curve analysis was performed. In addition, the correct size of PCR products was confirmed by DNA electrophoresis. Statistical analysis of the data was performed using two-way ANOVA and Students's *t* test.

RNA Interference. NIH3T3 cells were cultured in 6-well plates to ~50% confluence. Transfections were performed using DharmaFECT3 reagent (Dharmacon, Lafayette, CO) according to the manufacturer's protocol. The following Stealth RNA interference (RNAi) duplexes (Invitrogen, Carlsbad, CA) were used: UGGT, UAAUUAUACGCUCUCAGAACAGGGA; Sep15, UCCUGAGCGAGAAGUUGGAACGCAU; SelM, CCGAACUCGUGCUGUUAAGCCGAAA; SelS, GGAAGAUCUAAAUGCCCAAGUUGAA; SelT, AAGACUGACAGGAAAGAUGCUAUGU. As a control, Stealth RNAi negative control duplexes with medium GC content were used. 72 h after transfection cells were metabolically labeled with ^{75}Se for 24 h as described above and protein lysates were resolved by SDS−PAGE, followed by immunoblot and autoradiography. Sep15, SelM, and SelT-specific antibodies were previously developed in our laboratory, BiP-specific antibody was from Santa Cruz Biotechnology (Santa Cruz, CA), β-actin and SelS-specific antibodies were from Sigma (St. Louis, MO), UGGT-specific antibody was a kind gift from A. Parodi.

Gene Expression Analysis. Allen Brain Atlas was used to evaluate expression patterns for Sep15 and proteins involved in the ER quality control in the adult mouse brain (*20*). Gene expression data were visualized with an Allen Brain Atlas online tool. Relative gene expression was labeled on a discrete Red-Green-Blue color scale with increasing levels of expression from blue to red.

Transmission Electron Microscopy. C57BL/6J mice were given a single 1 μg/g of body weight intraperitoneal injection of a 0.05 mg/mL suspension of tunicamycin in 150 mM dextrose as described (*21*). 72 h after injection, the mice were euthanized by CO_2 asphyxiation, and livers were removed and fixed with 2.5% glutaraldehyde in Sorensen's buffer. Samples were postfixed in 1% osmium tetroxide, rinsed, and stained in saturated uranyl acetate. The samples were rinsed again, dehydrated in a graded ethanol series, embedded in Spurrs resin, and examined using a Hitachi H7500 transmission electron microscope (Hitachi, Pleasanton, CA). The care of animals was in accordance with the University of Nebraska and National Institutes of Health institutional guidelines.

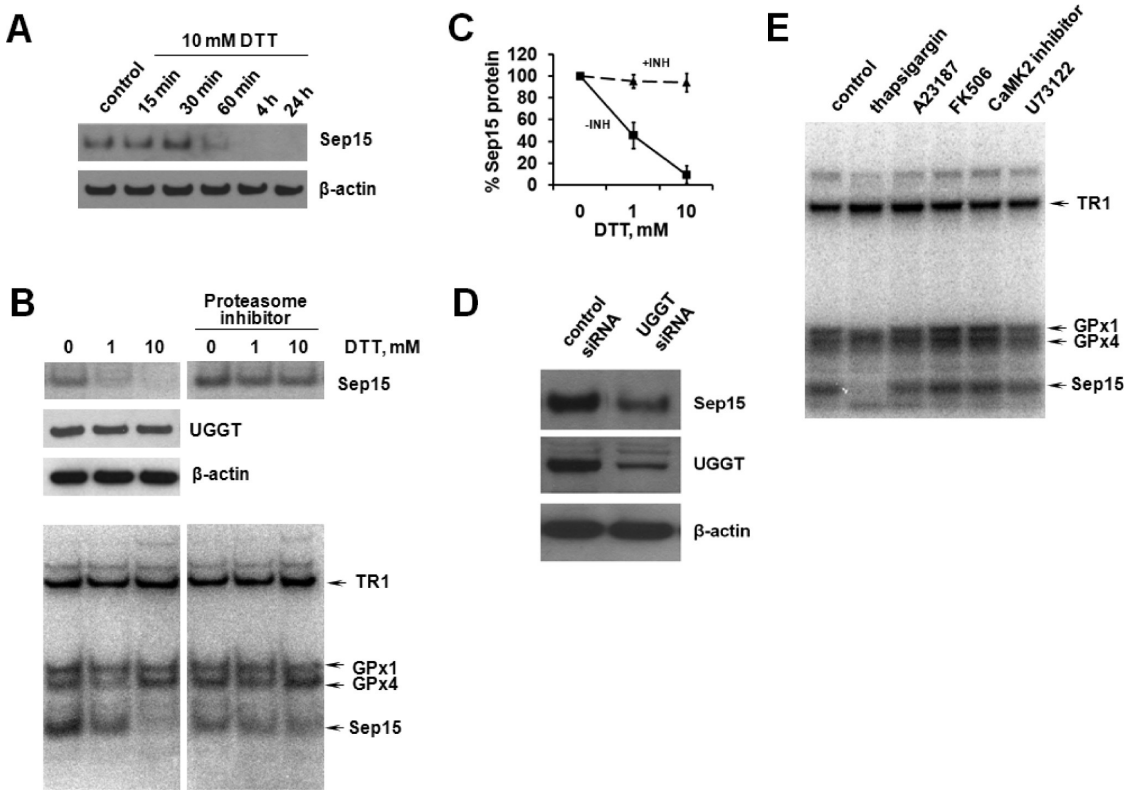

FIGURE 4: DTT and thapsigargin stimulate specific degradation of Sep15 by proteasomes. (A) Timing of DTT-dependent Sep15 degradation was analyzed by immunoblot analyses in NIH3T3 cells exposed to 10 mM DTT at various time intervals from 15 min to 24 h. (B) NIH3T3 cells were treated with indicated concentrations of DTT in the presence or in the absence of a 10 μM proteasome inhibitor Z-Leu-Leu-Leu-al. Expression of Sep15, UGGT, and β-actin were analyzed by immunoblot assays (upper panels). The extent of Sep15 degradation has been quantified and plotted in (C). Expression patterns of selenoproteins were assessed by labeling cells with ^{75}Se followed by SDS−PAGE and autoradiography (lower panels). Migration of Sep15 and major endogenous selenoproteins, thioredoxin reductase 1 (TR1), glutathione peroxidase 1 (GPx1), and glutathione peroxidase 4 (GPx4) is shown on the right. (C) Degradation of Sep15 by DTT in the presence (+*INH*) or absence (−*INH*) of proteasome inhibitor was quantified by densitometry. Results are represented as means ± SEM of three independent experiments. (D) Cells were transfected with UGGT-specific or control siRNA duplexes, and protein lysates were probed by immunoblot assays with polyclonal Sep15 and UGGT-specific antibodies, or monoclonal β-actin-specific antibody. (E) Cells were labeled with ^{75}Se followed by treatments with thapsigargin (5 μM), A23187 (5 μM), FK506 (500 nM), CaMK2 inhibitor (1 μM), or U73122 (1 μM) for 24 h. Selenoprotein expression patterns were analyzed by SDS−PAGE and autoradiography.

RESULTS

Sep15 is Differentially Regulated by ER Stress. To test if expression of Sep15 is affected by accumulation of unfolded proteins within the ER, we treated NIH3T3 cells with different ER stressors for 24 h. We used tunicamycin, an inhibitor of N-linked glycosylation in the ER (*22*), and brefA, which blocks transport of proteins from the ER to Golgi leading to an accumulation of proteins and ER overload (*23*). Both tunicamycin and brefA increased expression of Sep15 (Figure 1, panels A and B, respectively). To confirm activation of UPR by these treatments, we analyzed expression of BiP, an ER chaperone, which is also up-regulated by ER stress and is commonly used as an ER stress marker. In this experiment, BiP expression was induced by 100 ng/mL of tunicamycin and 1 μM brefA.

Thapsigargin and DTT are also known to cause ER stress. Thapsigargin inhibits transport of Ca^{2+} to the ER resulting in depletion of Ca^{2+} in the ER lumen (*24*). DTT affects the folding process by a different mechanism as it blocks formation of disulfide bonds in the ER preventing oxidative folding of membrane and secretory proteins (*25*). In contrast to the experiments involving brefA and tunicamycin, treatments with low concentrations of either thapsigargin or DTT did not up-regulate Sep15 protein expression. Moreover, at higher concentrations, these ER stressors decreased expression of Sep15 (Figure 1C,D). We also found that thapsigargin induced ER stress response and elevated expression of BiP at 100 nM, but levels of BiP protein were not changed when cells were treated with DTT for 24 h. To show that the UPR pathway is induced by DTT under these conditions, we analyzed the extent of *Xbp1* mRNA splicing, which is also used to monitor ER stress. Activation of the UPR pathway by DTT was confirmed by increased levels of spliced *Xbp1* mRNA (Figure 1E).

Depending on severity and timing of ER stress, UPR may either promote adaptation of cells by reducing protein load and increasing protein-folding capacity or it may initiate apoptosis in response to persistent ER stress (*26*). In our experiments, thapsigargin and DTT induced more rapid and robust ER stress compared to tunicamycin and brefA, which may explain degradation of Sep15 by these pharmacological agents. Treatment of cells with both thapsigargin and DTT resulted in increased levels of spliced *Xbp1* mRNA after 4 h, while tunicamycin and brefA up-regulated *Xbp1* mRNA splicing only after 24 h treatment (Figure 1E and Figure S1, Supporting Information). Moreover, viability of cells upon treatment with DTT was significantly decreased compared to tunicamycin treated cells suggesting that at high concentrations DTT activates a pro-apoptotic ER stress response (Figure S2, Supporting Information). Together, these results indicate that Sep15 is regulated by common ER stresses and its expression depends on the mechanism by which ER stress is induced.

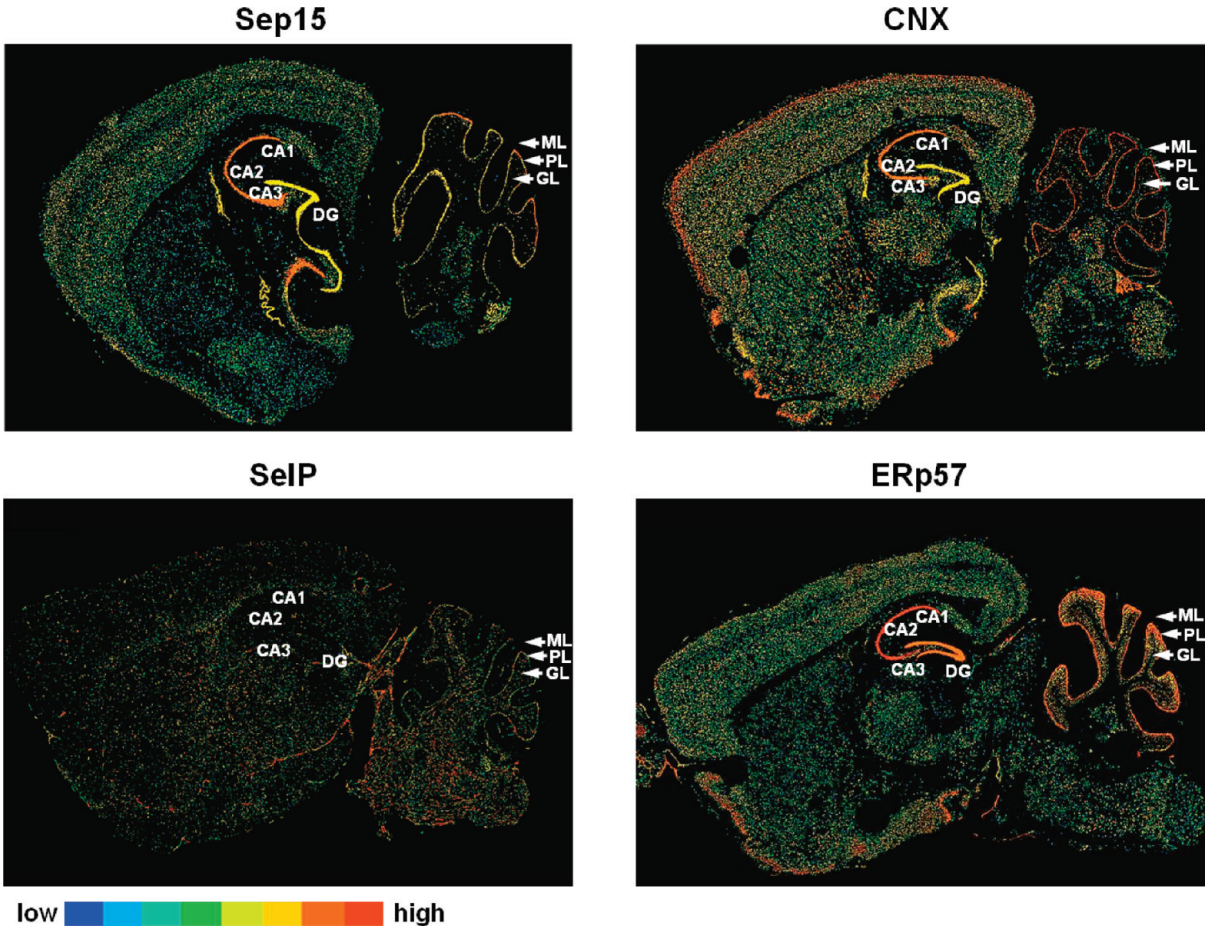

FIGURE 5: Expression profile of Sep15 selenoprotein in mouse brain. Expression pattern of Sep15 mRNA and those of the ER chaperone CNX and oxidoreductase ERp57, which are the major components of the quality control mechanism in the ER, were evaluated across 159 different brain regions. One representative image extracted from the same or close coronal plane positions is shown for each gene. Expression of selenoprotein SelP that is uniformly distributed throughout the brain structures is shown for comparison. CA1-CA3, Ammon's horn; DG, dentate gyrus; ML, molecular layer; PL, Purkinje layer; GL, granular layer. Relative gene expression is labeled on a discrete red−green−blue color scale with increasing level of expression from blue (low) to red (high).

Adaptive UPR Induced by Short-time Incubation with DTT Increases Expression of Sep15. DTT activates UPR by reducing disulfide bonds in proteins and perturbing protein folding in the ER. Prolonged incubation with DTT leads to acute ER stress, but it can be reversed by removing the reducing agent (Figure 2A). To assess whether Sep15 expression is changed during adaptation, we treated cells with different concentrations of DTT for 1 h followed by 8 h recovery (Figure 2B). In contrast to acute ER stress, adaptive UPR increased Sep15 expression when cells were preincubated with 0.1 and 1 mM DTT.

BrefA Induces Sep15 Expression at the Transcriptional Level. To determine whether Sep15 expression is affected by ER stress at the transcriptional level, we measured *Sep15* mRNA levels in mouse NIH3T3 cells treated with increasing concentrations of brefA. We found that after treatment with brefA, the expression of *Sep15* mRNA was increased 2-fold (Figure 3) confirming that the UPR signaling pathway activates Sep15 at the transcriptional level.

DTT Stimulates Rapid and Specific Degradation of Sep15 by Proteasomes. Induction of UPR in cultured cells is commonly achieved by supplementing media with pharmacological ER stressors for 12−24 h. In contrast to tunicamycin and brefA, DTT decreased Sep15 protein levels between 4 and 24 h, and this effect was already evident at 60 min (Figure 4A).

The observation that Sep15 levels were decreased by treatment with DTT raised the possibility that Sep15 may be posttranslationally regulated by this ER stressor. Sep15 does not possess a typical ER retention signal, and is kept in the ER through its interaction with UGGT, which is mediated by an N-terminal cysteine-rich domain of Sep15 (*16*). It is possible that DTT reduces disulfide bonds and compromises the structural integrity of the cysteine-rich domain disrupting interaction between Sep15 and UGGT. As a result, Sep15 protein may be degraded or secreted. To test this hypothesis, we treated cells with different concentrations of DTT in the presence or in the absence of a proteasome inhibitor (Figure 4B,C). In the absence of proteasome inhibitor, DTT degraded Sep15 protein. However, when proteasomal degradation was blocked, expression of Sep15 was not changed by treatment with DTT suggesting that DTT degrades Sep15 through the proteasome pathway. To test whether this effect is specific for Sep15, we metabolically labeled cells with radioactive selenium and analyzed expression of selenoproteins by autoradiography of SDS−PAGE gels. PhosphorImager analysis revealed that only Sep15 was degraded, whereas the levels of other major selenoproteins were not changed upon treatment with DTT. Moreover, down-regulation of UGGT by RNAi resulted in a decreased steady-state level of Sep15 (Figure 4D). This latter observation is also consistent with the idea that Sep15 is retained in the ER by interaction with its binding partner.

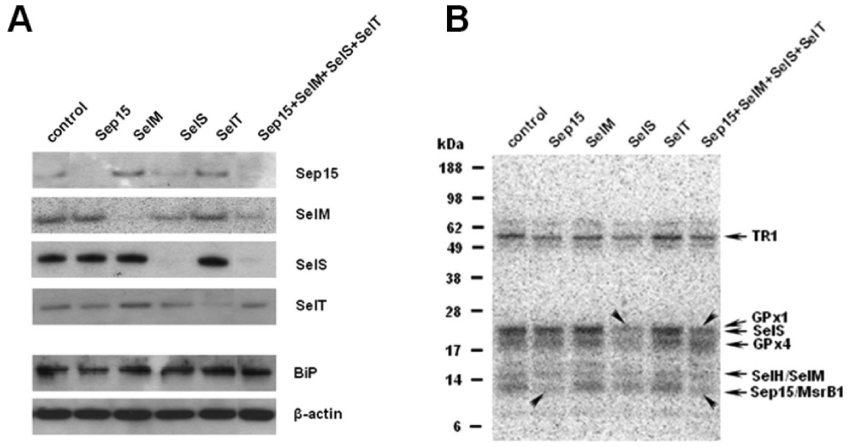

FIGURE 6: Down-regulation of Sep15 by siRNA does not induce UPR. NIH3T3 cells were transfected either with scrambled siRNA or Sep15, SelM, SelS, and SelT-specific siRNAs as indicated. (A) Protein lysates were probed by immunoblot analysis with Sep15, SelM, SelT and BiP-specific polyclonal, or SelS and β-actin-specific monoclonal antibodies. (B) Expression patterns of selenoproteins were assessed by labeling cells with ^{75}Se followed by SDS−PAGE and autoradiography. Migration of Sep15, SelM, SelS, and other major endogenous selenoproteins, thioredoxin reductase 1 (TR1), glutathione peroxidase 1 (GPx1), glutathione peroxidase 4 (GPx4), selenoprotein H (SelH), and methionine sulfoxide reductase B1 (MsrB1) is shown on the right.

Similarly to DTT, thapsigargin at high concentrations degraded Sep15 and this degradation was very specific as expression of the rest of selenoproteins was not changed. Moreover, treatments with stressors that affect intracellular Ca^{2+} homeostasis (Figure 4E) or other stress stimuli (Figure S3, Supporting Information) did not decrease Sep15 levels.

Expression Pattern of Sep15 in Mouse Brain. Previously, we utilized Allen Brain Atlas to characterize expression patterns of selenoproteins in the mouse brain (*27*). In this study, the expression pattern of Sep15 was compared with those of various components of the quality control machinery in the ER. Sep15 showed high levels of gene expression in neuronal cells of Ammon's horn (CA1-CA3) and dentate gyrus (DG) structures of hippocampus as well as in Purkinje cells of the cerebellar cortex (Figure 5). ERp57 and CNX, key proteins involved in quality control in the ER, had very similar expression profiles. The highest level of expression of these proteins was observed in CA1-CA3 and DG regions of hippocampus. In addition, elevated levels of CNX and ERp57 were present in cerebellum similar to Sep15, although their expression pattern was not uniform. In contrast to Sep15, expression of selenoprotein P (SelP), a Sec-containing protein responsible for transport and storage of selenium in the brain, was more homogeneous throughout the brain, consistent with astrocytic or oligodendroglial localization. The coexpression of Sep15 with ERp57 and CNX in brain neurons further links Sep15 to the folding processes involving these chaperones.

Decreased Levels of Sep15 do not Activate UPR. We further tested whether Sep15 deficiency leads to an accumulation of unfolded proteins and itself causes ER stress. RNAi was used to suppress Sep15 gene expression in mouse NIH3T3 and human HEK293 cell lines, and effective knockdown was confirmed by immunoblot assays and metabolic labeling with ^{75}Se (Figure 6 and Figure S4, Supporting Information). However, decreased levels of Sep15 did not lead to activation of UPR (as judged by expression of BiP protein), suggesting that the function of Sep15 may be compensated by other ER localized selenoproteins or other proteins. To further examine this possibility, we used RNAi to down-regulate SelM, a selenoprotein distantly related to Sep15, and two other ER-resident selenoproteins, SelS and SelT. Decreased levels of Sep15, even when combined with down-regulation of SelM, SelS, or SelT, did not increase expression of BiP. Similarly, down-regulation of individual ER selenoproteins did not induce BiP expression.

Sep15 is Induced by ER Stress in Vivo. Our data in the cell culture model of ER stress suggest that Sep15 expression is up-regulated through UPR signaling and might be required for protection of cells from stress. To examine whether Sep15 is induced by ER stress in vivo, C57BL/6J mice were challenged by intraperitoneal injection with tunicamycin, and the ER stress response in the liver was monitored by transmission electron microscopy (Figure 7A). Hepatocytes from animals challenged with tunicamycin showed significant distortion of ER morphology. In contrast to cells from vehicle treated mice, the ER in hepatocytes from mice injected with tunicamycin displayed increased fragmentation and took a form of dilated vesicles. Apparent dilation of the ER is consistent with the adaptation of cells to protein misfolding and ER overload caused by tunicamycin (*26*). Further expression analyses by immunoblot assays revealed that injection of tunicamycin activated UPR, as assessed by increased levels of BiP protein, and it also up-regulated Sep15 expression (Figure 7B). Thus, ER stress can also induce Sep15 expression in vivo.

DISCUSSION

The accumulation of unfolded or misfolded proteins in the ER results in activation of a protective mechanism known as the UPR. This signaling pathway allows cells to adapt to the ER stress by (i) temporary inhibition of protein synthesis to reduce the number of proteins entering the ER (*28*), (ii) transcriptional activation of genes encoding components of ERAD that eliminate misfolded or unassembled proteins from the ER (*29*−*31*), and (iii) increased expression of chaperones and thiol-disulfide oxidoreductases involved in protein folding in the ER (*32*−*34*). A recently identified Sec-containing oxidoreductase Sep15, has been shown to reside in the ER (*35*). This protein forms a 1:1 complex with UGGT, suggesting a possibility that it functions as a protein disulfide isomerase that targets a set of glycoproteins that are substrates of UGGT (*15*).

We hypothesized that expression of Sep15 may also be regulated in response to an accumulation of unfolded proteins in the ER. To test this hypothesis, we induced ER stress in mouse

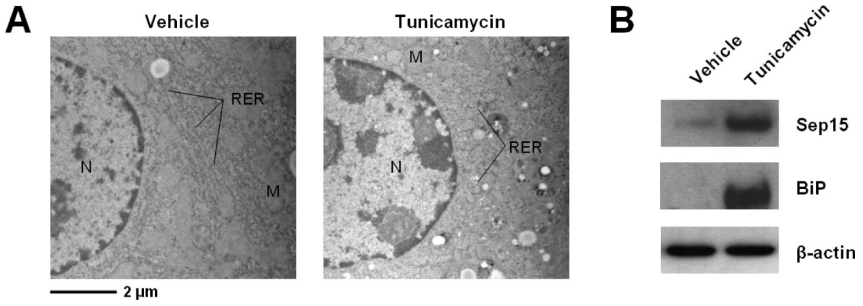

FIGURE 7: Expression of Sep15 is induced by ER stress in vivo. (A) Electron microscopy. C57BL/6J mice were injected intraperitoneally with tunicamycin (1 μg/g body weight) or vehicle. 72 h after injection the mice were euthanized, livers were fixed in 2.5% glutaraldehyde and analyzed by transmission electron microscopy. Mitochondria (M), nuclei (N), and rough ER (RER) are indicated. (B) Western blot analysis. Protein lysates from livers were probed by immunoblot assays with polyclonal antibodies specific for Sep15 and BiP, or monoclonal β-actin antibody as a loading control.

NIH3T3 cells by four different pharmacological agents, namely, tunicamycin, brefA, thapsigargin, and DTT, that perturb protein folding by different mechanisms. All of the ER stressors, which were used in this study, lead to an accumulation of unfolded proteins in the ER and activation of UPR. However, expression of Sep15 was increased only by treatments with tunicamycin and brefA during a 24 h treatment. In contrast, thapsigargin and DTT stimulated rapid degradation of Sep15. In order to explain the observed discrepancy, we analyzed the kinetics of induction of UPR by different ER stressors. Our results demonstrate that thapsigargin and DTT induced more rapid and robust ER stress, as judged by splicing of *Xbp1* mRNA, compared to brefA and tunicamycin. The different kinetics of UPR activation by these stressors depends on the mechanism by which ER stress is induced. Tunicamycin and brefA rely upon protein biosyntheses to exert their effects and disrupt protein folding less robustly. In contrast, DTT and thapsigargin induce protein misfolding in the ER independently of protein synthesis by reducing disulfide bonds in existing proteins, or by inducing rapid flux of calcium from the ER, respectively.

We also found that treatment of cells with 10 mM DTT led to a specific proteasomal degradation of the Sep15 protein. Sep15 is normally retained in the ER by interaction with its binding partner UGGT through its N-terminal cysteine-rich domain (*16*). However, treatment of cells with a reducing agent could lead to conformational changes in this domain and disrupt interaction between Sep15 and UGGT. As a result, Sep15 would be targeted for degradation by proteasomes. In the case of thapsigargin, Sep15 levels are also most likely regulated at the posttranslational level. It was previously reported that the catalytic activity of UGGT is dependent upon the presence of Ca^{2+} ions (*36*). Depletion of Ca^{2+} in the ER by thapsigargin may prevent the interaction between Sep15 and UGGT that would ultimately lead to degradation of the released protein.

A possible role of Sep15 in formation of disulfide bonds and quality control in the ER is further supported by expression profiles of this protein in mouse brain. We took advantage of the recently released expression atlas of the mouse brain (*20*) to compare the expression patterns of Sep15 and proteins involved in quality control in the ER. Strikingly, expression of Sep15 colocalized with expression of CNX and ERp57 in the neuronal cells.

If Sep15 assists oxidative folding and structural maturation of proteins, one would expect that down-regulation of Sep15 would prevent folding of proteins targeted by UGGT. However, our data demonstrate that decreased levels of Sep15 do not induce ER stress. In addition, we sought to determine whether other selenoproteins localized to the ER could compensate for Sep15 function. Selenoprotein SelS, also known as VIMP (for VCP-interacting membrane protein), has been recently shown to interact with Derlin-1 and p97/VCP, which constitute the ER-associated degradation machinery (*37*). Depletion of SelS is expected to compromise efficiency of ERAD leading to a diminished rate of misfolded protein removal from the ER and aggravation of ER stress. Decreased levels of Sep15 combined with siRNA-based knockdown of SelS as well as SelM and SelT, two other ER-resident thioredoxin-like selenoproteins, did not lead to ER stress suggesting that other factors/mechanisms may compensate for Sep15 function.

The fact that UGGT deficiency leads to a delayed release of a specific group of glycoproteins from CNX suggested that these glycoproteins require UGGT activity or the activity of a UGGT-associated protein to complete their folding needed for substrate dissociation from CNX (*13*). It is possible that Sep15 which forms a tight complex with UGGT may assist in oxidative folding of this group of CNX substrates. The lack of detectable ER stress in Sep15-deficient cells is also consistent with this observation. Taken together, our data further support the role of Sep15 in protein folding and suggest that it may assist maturation of a restricted group of N-glycosylated proteins.

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SUPPORTING INFORMATION AVAILABLE

Extent of *Xbp1* mRNA splicing in cells treated with tunicamycin, brefA, thapsigargin, and DTT (Figure S1), viability of NIH3T3 cells upon treatment with tunicamycin and DTT (Figure S2), specific degradation of Sep15 by thapsigargin (Figure S3), and down-regulation of Sep15 by RNAi in human HEK293 cells (Figure S4). This material is available free of charge via the Internet at http://pubs.acs.org.

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