ERp57 modulation of STAT3 signaling from the lumen of the Endoplasmic Reticulum

Helen Coe1,2, Joanna Jung1, Jody Groenendyk1, Daniel Prins1, and Marek Michalak1,2

From the Departments of 1Biochemistry and 2Pediatrics, University of Alberta, Edmonton, Alberta, Canada T6G 2H7

Running head: ERp57 and STAT3 regulation

Address correspondence to: Marek Michalak, Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada T6G 2S7, Tel: 780-492-2256, Fax: 780-492-0886, E-mail: marek.michalak@ualberta.ca

ERp57 is an endoplasmic reticulum (ER) resident thiol disulfide oxidoreductase. Using the gene trap technique, we have created an ERp57-deficient mouse model. Targeted deletion of the Pdia3 gene, which encodes ERp57, in mice is embryonic lethal at embryonic day (E) 13.5. β-galactosidase reporter gene analysis revealed that ERp57 is expressed early on in blastocyst formation with the highest expression in the inner cell mass. In early stages of mouse embryonic development (E11.5) there was a relatively low level of expression of ERp57. As the embryos developed, ERp57 became highly expressed in both the brain and the lungs (E15.5 and E18.5). The absence of ERp57 had no impact on ER morphology; expression of ER associated chaperones and folding enzymes, ER stress, or apoptosis. ERp57 has been reported to interact with STAT3 (signal transducers and activators of transcription)-DNA complexes. We show here that STAT3-dependent signaling is increased in the absence of ERp57 and this can be rescued by expression of ER targeted ERp57 but not by cytoplasmic targeted protein, indicating that ERp57 affects STAT3 signaling from the lumen of the ER. ERp57 effects on STAT3 signaling are enhanced by ER luminal complex formation between ERp57 and calreticulin. In conclusion, we show that ERp57 deficiency in mouse is embryonic lethal at E13.5 and ERp57-dependent modulation of STAT3 signaling may contribute to this phenotype.

The endoplasmic reticulum (ER) is involved in many cellular functions including protein synthesis and modification, regulation of Ca2+ homeostasis, phospholipid and steroid synthesis, and regulation of the response to cellular stress (1-3). To carry out these diverse functions, the ER is equipped with many chaperone proteins and folding enzymes (3). For example, calreticulin, calnexin, and the oxidoreductase ERp57 are components of the folding machinery involved in the quality control of newly synthesized glycoproteins (3). ERp57 forms complexes with calnexin and calreticulin to assist in chaperone functions to ensure that newly synthesized proteins are correctly folded (3). Targeted deletion of ERp57 in B cells in mice results in normal B cell development and proliferation as well as antibody production (4). However, there is aberrant assembly of the peptide loading complex indicating that ERp57 is involved in the assembly of the peptide loading complex and this protein contributes both qualitatively and quantitatively to major histocompatibility complex (MHC) class I antigen presentation in vivo (4). Interestingly, small interfering RNA (siRNA) studies also demonstrate that ERp57 might be critical for oxidative folding of immunoglobulin heavy chain but not important for peptide loading of class I molecules (5).

Aside from its role as a folding enzyme in quality control in the secretory pathway, ERp57, also known as glucose-regulated protein-58 or Grp58, (6-13) has been reported to affect STAT3 (signal transducers and activators of transcription) signaling via interaction with STAT3 (6,14-17). STATs are a family of cytoplasmic proteins with SH2 (Src Homology-2) domains that act as signal messengers and transcription factors as a part of the Janus kinase (JAK)-STAT pathway (18). Upon activation, STAT proteins become phosphorylated on a specific tyrosine residue by activated JAK kinases and subsequently dimerize and translocate to the nucleus where they bind to specific DNA sequences to regulate the expression of target genes (18,19). Among the STAT proteins, STAT3 has been implicated in transduction of the cellular signals that are involved in the regulation of cardiac growth, development, and hypertrophy (20). Although it is not clear how STAT3 activity is negatively regulated by ER resident ERp57, it has been reported that ERp57 may sequester the inactive and active STAT3 preventing its interaction with DNA and consequently activation of STAT3-dependent genes (6,12).
In this study we created ERp57-deficient mice and ERp57−/− cell lines. ERp57 deficiency was embryonic lethal at embryonic day 13.5 (E13.5) and ERp57-deficient cells had significantly increased STAT3 activity. We showed that the ER, but not cytosolic, form of ERp57 is responsible for inhibition of STAT3 activity. Furthermore, ERp57-dependent modulation of STAT3 was enhanced by ER luminal interactions between ERp57 and calreticulin. Our results suggest that, in vivo, ERp57 and STAT3 may not interact and that the observed modulation of STAT3 activity may be due to ERp57-dependent signalling from the ER.

**EXPERIMENTAL PROCEDURES**

**Generation of ERp57-Deficient Mice** - Gene trapping with the trap vector pGT1Tmpls was used to generate the Pdia3 gene disrupted embryonic stem cells from the Gene Trap Resource at http://baygenomics.ucsf.edu (BayGenomics, University of San Francisco, San Francisco, California). The Pdia3 gene encodes ERp57. Parental cell lines (CGR8 and E14Tg2A) were established from delayed blastocysts on gelatinized tissue culture dishes in embryonic stem cell medium containing leukemia inhibitory factor (LIF) (21). Embryonic stem cells were cultured in a media containing Glasgow minimal essential medium (GMEM), 2 mM glutamine, 1 mM sodium pyruvate, non-essential amino acids, 10% fetal calf serum, 100 nM leukemia inhibitory factor (LIF) (21). Embryonic stem cells were microinjected into 3.5 day old C57BL/6J blastocysts to generate chimeric mice (23). Chimeric males were analyzed for germline transmission by mating with C57BL/6J females, and the progeny was identified by PCR analysis, β-galactosidase staining of blastocysts, and Western blot analysis. All animal experimental procedures were approved by the Animal Welfare Program at the Research Ethics Office, University of Alberta and conformed to relevant regulatory standards.

**Genotype Analysis of ERp57-Deficient Mice** - Genomic DNA from heterozygous mice (carrying the LacZ gene) was isolated using DNeasy Blood & Tissue kit (Invitrogen). Genomic DNA was purified using a PCR purification kit (Qiagen), and then DNA fragments were ligated using the Rapid DNA Ligation Kit (Boehringer). First, PCR amplification was performed using primers specific to the inserted vector: INVF1, 5′-GTTCCTAAGCATACAGGCCAG-3′; and INVR1, 5′-AAGGCCATACAAACGACGAGGC-3′. The product from the PCR reaction was used as a template for second PCR with specific (nested) primers: INVF2, 5′-CGAGCGTGACACACGATGC-3′; and INVR2 5′-CGAGCGTGACACACGATGC-3′. Products of the inverse PCR-driven amplification were analyzed by agarose gel electrophoresis, purified using a gel purification kit (Qiagen), and sequenced. Once the integration site was identified, we designed a protocol for genotyping wild-type, heterozygote and homozygote ERp57-deficient embryos (Fig. 1). Embryos were harvested at embryonic days 10.5-15.5 followed by the isolation of genomic DNA. The following PCR primers were used for genotyping wild-type embryos: forward primer (F2) 5′-GGACAGTTTGGAGCCTGCCCC-3′ (hybridizes to the intron within the insertion of the vector site) and reverse primer (R3) 5′-TCTCCATTATCATCCTGTACTCC-3′ (hybridizes to intron 4 after the vector insertion site). To identify heterozygous embryos the following primers were used: forward primer (F1) 5′-TCAAGGCGAGTTACATGATCCC-3′ (hybridizes to the end of the inserted vector) and reverse primer R3.

**Histological Analysis** - Blastocysts were harvested at 3 days post-coitum and whole-mount β-galactosidase staining of blastocysts was performed. Blastocysts were washed with phosphate buffered saline (PBS), fixed in 3.7% paraformaldehyde in PBS for 30 min, then washed with PBS. Blastocysts were then incubated for 1 h in freshly prepared staining solution in 10 ml of PBS containing 2 mM MgCl₂, 0.01% sodium deoxycholate, 0.02% NP40, and 0.1% 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-gal) dissolved in dimethyformamide (24). Photographs of blastocysts were taken using a Nikon Coolpix 995 camera attached to a Nikon eclipse TS100 microscope with a 10x magnification objective. β-galactosidase, hematoxylin, and eosin staining of wild-type and ERp57−/− embryos was carried out as described previously (2). β-galactosidase stained slides were counterstained with eosin (0.1% eosin in 0.1% acetic acid.). Embryos were viewed with the Nikon Coolscan IV.

**Cell Culture, Plasmid DNA, DNA Cloning and Lentivirus Transduction** - Mouse embryonic fibroblasts were isolated from day-11 wild-type and ERp57-deficient embryos, immortalized and maintained in culture as described previously (23). When indicated, cells were treated with 1 μM thapsigargin or 2.5 μM brefeldin A for 16 hours.

cDNAs encoding full-length ERp57 or encoding mature ERp57 with no signal sequence were isolated from a mouse brain cDNA library using Gateway Cloning.
The following forward DNA primers were used for amplification of full length ERp57 and no signal sequence ERp57: 5’-GGGGACAAAGTTTGTAACAAAAAGCAGGCTATAC CATCCGGTTCAGGCTAGCT-3’ and 5’-GGGGACAAAGTTTGTAACAAAAAGCAGGCTATAC CATCGGATGTGGGACTGACGGAGA -3’, respectively. The forward primers contain an attB1 recombination site (bold), Kozak sequence for expression in mammalian cells (italics), and gene specific nucleotides (underlined). The same reverse primer was used for the synthesis of cDNAs encoding full-length and no signal sequence ERp57: 5’-GGGGACACTTTGTAACAGAAAGCTGGTGCCCT-3’. The reverse primer contained an attB2 recombination site (bold), Kozak sequence for expression in mammalian cells (italics), and gene specific nucleotides (underlined). Both forward and reverse primers required four guanine residues at the 5’ end. First, a BP clonase reaction was carried out using BP Clonase Enzyme Mix to insert residues at the 5’ end. A recombination reaction carried out as recommended by the manufacturer to generate entry clone vectors. A recombination reaction was carried out using BP Clonase Enzyme Mix to insert cDNA encoding full-length ERp57 or no signal sequence ERp57 with the promoter EF1α (cellular polypeptide chain elongation factor 1 alpha) into the destination lentiviral vector 2K7bsd (containing a blasticidine resistance gene for cell selection). Expression vectors p2K7ERp57 and p2K7ERp57cyt contained cDNA encoding full-length ERp57 and no-signal sequence ERp57, respectively. Lentiviral transduction techniques were used to generate ERp57Δ- and wild-type fibroblast-derived cells lines stably expressing recombinant proteins ERp57 or no-signal sequence ERp57 (25). Protein from bulk cell population was harvested with RIPA buffer containing 50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, and protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride [PMSF], 0.5 mM benzamidine, 0.05 µg/ml aprotonin, 0.025 µg/ml phosphomidone, 0.05 µg/ml N-tosyl-Lys-chloromethylketone [TLCK], 0.05 µg/ml 4-amidinophenyl)-methanesulfonyl fluoride hydrochloride monohydrate [APMSF], 0.05 µg/ml (2S,3S)-3-(N-f3dcarbamoyl)oxirane-2-carboxylic acid [E-64], 0.025 µg/ml leupeptin, and 0.01 µg/ml pepstatin) (26). Expression of full-length ER targeted ERp57 and cytoplasmically targeted ERp57 (no signal sequence ERp57) was monitored by Western blot analysis (2,25,27). ERp57-deficient cells expressing full-length ERp57 were denoted ERp57Δ-ERp57ER and cells expressing no signal sequence-cytoplasmic ERp57 were denoted ERp57Δ-ERp57cyt.

Reverse Transcription (RT)-PCR - Total RNA was isolated from different tissues using TRizol Reagent (Invitrogen). cDNA was synthesized using Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Invitrogen) and amplified with Taq polymerase (Sigma) using the following primers: for ERp57, forward primer 5’-GGACAGTTTTGAGCTGAGGATCC-3’ and reverse primer 5’-GTCTTCCATTATCAGTACTC-3’; and reverse primer 5’-CTCTCCATTATCAGTACTC-3’; and reverse primer 5’-AGTCTTCCAATCTGGCATCC-3’, for glyceraldehyde 3-phosphate dehydrogenase (GAPDH), SDS-Polyacrylamide Gel Electrophoresis (PAGE) and Western Blot Analysis - Whole cell lysates from wild-type, ERp57Δ-, ERp57Δ-ERp57ER and ERp57Δ-ERp57cyt mouse embryonic fibroblasts were isolated as described previously (2). Twenty µg of protein was separated by SDS-PAGE (10% acrylamide), transferred to nitrocellulose and probed with specific antibodies (28). Antibodies used were rabbit-anti-Grp78/BiP at a dilution of 1:1000 (StressGen), rabbit-anti-calnexin at a dilution of 1:1000 (StressGen), goat-anti-calreticulin at a dilution of 1:300, rabbit-anti-ERp57 at a dilution of 1:1000, rabbit-anti-protein disulfide isomerase (PDI) at a dilution of 1:1000, rabbit-anti-ERp41 at a dilution of 1:1000, rabbit-anti-ERp54 at a dilution of 1:1000, and rabbit-anti-β-tubulin (Abcam) at a dilution of 1:1000 (2,29).

For detection of phosphorylated-STAT3 (Y705-phospho STAT3), ERp57Δ-, ERp57Δ-ERp57ER, and ERp57Δ-ERp57cyt cells were grown to confluency and treated with 2 mM Na3VO4 for 20 minutes prior to harvesting. Cells were washed with PBS containing 4 mM Na2VO4 and harvested in RIPA buffer Thirty µg of protein was separated by SDS-PAGE (10% acrylamide) and transferred to nitrocellulose membrane (28). The nitrocellulose membrane was washed with 25 ml of TBS buffer containing 20 mM Tris (pH 7.6), and 150 mM NaCl, and blocked for 1 h in TBS containing 5% milk and 0.1% Tween 20. Membranes were probed with rabbit-anti-phospho-STAT3 (Y705) antibodies (Cell Signalling) or anti-STAT3 (Cell Signalling) at 1:1000 dilutions overnight at 4°C. Secondary antibodies used were goat-anti-rabbit (1:10,000 or 1:15,000 for anti-phospho-STAT3 antibodies) (Cell Signalling) and rabbit-anti-goat (1:10,000 for anti-STAT3 antibodies) (Cell Signalling). Blots were developed using a chemiluminescent system (28).
Immunohistochemistry and Electron Microscopy - For immunostaining, ERp57\(^{-/-}\), ERp57\(^{-/-}\)-ERp57\(_{ER}\), and ERp57\(^{-/-}\)-ERp57\(_{cyt}\) mouse embryonic fibroblasts were grown on glass coverslips. Cells were fixed with 3.7% paraformaldehyde in PBS for 20 min at room temperature (2). For immunostaining of ERp57, cells were permeabilized with 0.1% Triton-X100 in PBS for 20 min at room temperature and washed twice with PBS. Cells were incubated in PBS containing 1% bovine serum albumin (BSA) for 30 min at room temperature and then incubated with anti-ERp57 (1:100) in PBS containing 1% BSA for 1h at room temperature. The secondary antibody used was rabbit conjugated-fluorescein isothiocyanate (FITC) (Invitrogen) at a dilution of 1:1000 in PBS containing 1% BSA for 45 min at room temperature (30). Immunostaining with anti-calnexin and anti-calreticulin antibodies was carried out as described previously (28). All coverslips were co-stained with Alexa Fluor 546-Concanavalin A at a dilution of 1:1000 (Sigma). The coverslips were mounted onto glass slides and fluorescent signals visualized using spinning disk confocal microscopy (WaveFX from Quorum Technologies, Guelph, Canada) set up on an Olympus IX-81 inverted microscope (Olympus, Markham, Canada). Images were acquired using Volocity (Improvision) through the EMCCD camera (Hamamatsu, Japan). The fluorescent dyes, FITC and Texas Red, were excited by a 561 nm laser line, respectively (Spectral Applied Research, Richmond Hill Canada). Z-slices (0.25 µm) were acquired using Velocity (Improvision) through the cells using a piezo z-stage (Applied Scientific Instrumentation, Eugene, USA.)

For electron microscopy analysis, cells were harvested, spun down, and fixed at 4°C for 4 hours in a freshly prepared solution containing 2.5% glutaraldehyde and 2% paraformaldehyde in 100 mM cacodylate, pH 7.2 (31). Samples were processed for electron microscopy and examined with a Hitachi Transmission Electron Microscope H-7000.

Subcellular fractionation - Wild-type, ERp57\(^{-/-}\), and ERp57\(^{-/-}\)-ERp57\(_{cyt}\) cells harvested in a buffer containing of 250 mM Sucrose, 20 mM Hepes, pH 7.0, 10 mM KCl, 1.5 mM MgCl\(_2\), 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 200 µM PMSF, 100 µM benzamidine, and protease inhibitors (26). The cellular suspension was incubated on ice for 10 minutes then subjected to 40 strokes with a dounce homogenizer followed by a 20 min incubation on ice. The sample was centrifuged at 720 xg for 5 min and the supernatant containing cytoplasm, mitochondria and membranes was centrifuged at 10,000 xg for 10 min. The supernatant containing cytoplasm and membranes was then subjected to a high speed centrifuge at 100,000 xg for 1 hour to separate the cytoplasmic fraction from the pellet containing ER-enriched membrane vesicles. Membrane pellet was suspended in 1 ml of RIPA buffer containing 50 mM Tris, pH 7.2, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Tx-100, 0.1% SDS, 0.5% sodium deoxycholate, 200 µM PMSF, 100 µM benzamidine, and protease inhibitors (26). Prior to SDS-PAGE proteins were precipitated with ice cold acetone. Proteins were transferred to nitrocellulose membrane followed by Western blot analysis with rabbit anti-ERp57 antibodies at a dilution of 1:1000.

FACS analysis - Samples were analyzed on BD FACScan single laser flow cytometry (BD Bioscience, San Diego) equipped with 488 nm filter. Data was collected from 10,000 cells and analyzed using CellQuest software. Cells at confluency of 80-90% were harvested by scraping into PBS, re-suspended in 100 µl of 0.1% formaldehyde in PBS and incubate for 30 min with rabbit anti-ERp57 antibodies at the concentration 1:150. Pelleted cells were then washed 3 times with PBS containing 2% FBS followed by addition of FITC label anti-rabbit (ALEXA Fluor 488 Invitrogen) secondary antibody. Cells were washed 3 times with PBS and FBS once with PBS alone. Staining with secondary antibodies alone was used as a negative control. All experiments were carried out at least three times.

Luciferase Reporter Gene Assay - Generation of crt\(^{-/-}\), crt\(^{-/-}\)-CRT\(_{G242A}\), and crt\(^{-/-}\)-CRT\(_{E234R}\) mouse embryonic fibroblasts was described previously (32). ERp57\(^{-/-}\) and ERp57\(^{-/-}\)-ERp57\(_{ER}\) cells were transfected with ER stress luciferase reporter vector [pRL-XFL, a generous gift from Dr. R.J. Kaufman (33)] as described previously (2). Twenty four hours after transfection, cells were treated with thapsigargin (1 µM) or H\(_2\)O\(_2\) (50 µM) for 16 hours. ERp57\(^{-/-}\), ERp57\(^{-/-}\)-ERp57\(_{ER}\), ERp57\(^{-/-}\)-ERp57\(_{cyt}\), crt\(^{-/-}\)-CRT, crt\(^{-/-}\)-CRT\(_{G242A}\), and crt\(^{-/-}\)-CRT\(_{E234R}\) cells were co-transfected with a luciferase reporter gene under control of the STAT3 promoter [pLucTKS3, a kind gift from Dr. J. Jung and was described previously (34,35)] with Renilla luciferase reporter at a 50:1 ratio, respectively (2). After 48 hours cells were harvested, lysed, and collected in Passive Lysis Buffer (Promega) followed by measurement
of luciferase activity using the Dual-Luciferase Assay Kit (Promega) and a Berthold-Lumat 9507 luminometer (2). Relative light units (RLUs) were normalized to the Renilla luciferase under the control of the Cytomegalovirus (CMV) promoter (2,33). Four independent transfection experiments were carried. Statistics were performed using two-sample unpaired t-tests were carried out.

Miscellaneous Procedures - Protein concentration was measured spectrophotometrically using a Bio-Rad procedure (2). Ca\(^{2+}\) fluorescence measurements were carried out using 0.5 \(\mu\)M Fura2-AM (22). Cells were treated with 600 nM bradykinin and 300 nM thapsigargin (36). Each trial was calibrated with 1 \(\mu\)M ionomycin and 4 mM CaCl\(_2\) to obtain a maximum value and with 30 mM ethylene glycol tetraacetic acid (EGTA), 25 mM Tris-HCl, pH 7.4, and 0.4 % Triton X-100 to obtain a minimum value. Fluorescence was measured at \(\lambda_{ex}=340\) nm and \(\lambda_{em}=380\) nm with \(\lambda_{em}=510\) nm. Ca\(^{2+}\) concentrations were calculated from the fluorescence values.

RESULTS

**ERp57 Deficiency is Embryonic Lethal** - Figure 1A summarizes the results of the gene trapping strategy used to generate the ERp57-deficient mice. The Pdia3 gene, which encodes ERp57, was interrupted between introns 4 and 5 (nucleotides 14083-14084) with the pGT1.8TM cassette containing neomycin resistance and \(\beta\)-galactosidase genes (Fig. 1A). PCR analysis of genomic DNA using the specific sets of primers depicted in Figure 1A allowed for determination of the genotypes of mice. Analysis of wild-type mice showed only a 797 base pair (bp) DNA product corresponding to the wild-type allele (Fig. 1B, primers F2 and R3) and no DNA product with primers F1 and R3 (Fig. 1B). As expected, ERp57\(^{-/-}\) mice showed a 1800 bp DNA product with primers F2 and R3 and no product with primers F1 and R3 (Fig. 1B). PCR-driven amplification of genomic DNA isolated from ERp57\(^{+/+}\) mice produced both the 797 bp and the 1800 bp DNA products, indicating the presence of one copy of the wild-type allele and one copy of the targeted knock-out allele (Fig. 1B). Western blot analysis revealed that interruption of both alleles of the ERp57 gene resulted in no detectable expression of ERp57 protein (Fig. 1C).

Chimeric mice were crossed with wild-type C57BL6 females to generate the first generation of heterozygotes. ERp57\(^{+/+}\) B6/CD-1 mice were indistinguishable from wild-type animals. ERp57\(^{+/+}\) males were intercrossed to ERp57\(^{+/+}\) females to generate homozygote (ERp57\(^{-/-}\)) gene knockout mice. We were unable to obtain any viable ERp57\(^{-/-}\) pups from this cross. Viable embryos were obtained at E13 or earlier (Table 1). Analysis of embryos at or after E13.5 showed a deficiency in the number of ERp57\(^{-/-}\) embryos indicating that a significant fraction of ERp57\(^{-/-}\) embryos died earlier than E13.5. We concluded that the ERp57 gene knockout was embryonic lethal and that ERp57 is essential for survival.

**Developmental Activation of the ERp57 Gene** - Histological analysis of E12.5 ERp57\(^{-/-}\) embryos revealed that although there were no obvious gross morphological differences between ERp57\(^{-/-}\) and wild-type embryos, the ERp57\(^{-/-}\) embryos were markedly smaller than wild-type (Fig. 1D). To gain additional insight into the potential role of ERp57 during embryonic development, we carried out \(\beta\)-galactosidase reporter gene analysis. The transgenic animals used in this study were generated by insertion of a gene cassette containing the \(\beta\)-galactosidase reporter gene (Fig. 1A). To quantify transcriptional activation of the ERp57 gene we monitored expression of the \(\beta\)-galactosidase gene to evaluate ERp57 promoter activity in blastocysts and during embryonic development. Figure 2A shows high expression and activity of \(\beta\)-galactosidase in blastocysts 3 days post-coitum indicating high expression of ERp57 during the early stages of blastocyst formation. High activation of the ERp57 gene was confined to the inner cell mass (Fig. 2A, icm) with relatively decreased activation of the gene in the blastocoel (Fig. 2A, b) and trophoblast (Fig. 2A, t). Next, ERp57\(^{-/-}\) and wild-type embryos were harvested at different gestational stages and stained for \(\beta\)-galactosidase activity. Activation of the ERp57 gene, as reported by high activity of \(\beta\)-galactosidase, was observed as early as E13.5 (Fig. 2B). At E13.5 there was high activation in the lung and vertebra but a relatively low activity of the gene in the liver and the heart (Fig. 2B). At E15.5 \(\beta\)-galactosidase reporter gene activity was the highest in the lungs with elevated activation also observed in the liver, vertebra and intestine (Fig. 2C). However, the activity was strikingly absent in the heart, thymus, and neurological tissue including the brain (Fig. 2C). At the later stages of embryonic development (E18.5), the pattern of activation of the ERp57 gene significantly changed. Low activity of the ERp57 promoter was maintained in the heart, thymus, and skeletal muscle and high activity was observed in the lungs and liver (Fig. 2D). At E18.5 we now observed a significant increase in the activation of the ERp57 gene in the brain which was absent in the E13.5 (Fig. 2D). These data suggest that ERp57 may play a direct role during embryonic development, specifically in lung, liver, and
vertebrae and, in the later developmental stages, in the brain.

The ER in ERp57-Deficient Mouse Fibroblasts - To determine the effects of ERp57 deficiency on ER functions, we isolated mouse embryonic fibroblasts from ERp57-deficient and wild-type embryos. Some of the ERp57-deficient cells were transfected with expression vectors encoding full length, ER-targeted ERp57 or cytoplasm-targeted ERp57 with no signal sequence and were designated ERp57/ER or ERp57/ERp57, respectively. As expected, Western blot analysis revealed that wild-type, ERp57/ERp57ER or ERp57/ERp57ER cells contained immunoreactive ERp57 (Fig. 3A). Figure 3B shows that wild-type and ERp57/57ER cells expressed ERp57 and that the protein was localized to an ER-like network. As expected, ERp57 was localized to the cytoplasmic compartment in ERp57-deficient cells expressing ERp57 without signal sequence (Fig. 3B, ERp57/-ERp57ER). Cytoplasmic localization of ERp57 without signal sequence (ERp57ER) was further confirmed by biochemical fractionation of ERp57-deficient cells expressing ERp57 without signal sequence (ERp57/ERp57ER) (Fig. 3C). Finally, we carried out FACS analysis of different cell lines and showed that there was a small level of immunoreactive ERp57 detected on cell surface of cell lines used in this study (Fig. 3D).

Morphologically, the ER appeared intact in all cell lines as judged by staining with Texas Red conjugated Concanavalin A, with antibodies raised against calreticulin and calnexin, or by electron microscopy analysis (Fig. 3B and E). Western blot analysis demonstrated that there was no significant differences in the expression of ER chaperone proteins calreticulin, calnexin, Grp78/BiP, and Grp94 in the absence of ERp57 (Fig. 3F). Next we examined the impact of the absence of ERp57 on other oxidoreductase folding enzymes family members. Again there was no significant change in expression of PDI and PDI-like family of protein ERP19 in the absence of ERp57 (Fig. 3F). Slight increase in the level of ERP41 and decrease in the level of ERP54 was seen in the absence of ERp57 (Fig. 3F). Thus, the absence of ERp57 had no major impact on the expression of other ER chaperones or folding enzymes.

ER Ca²⁺ Homeostasis in the Absence of ERp57 - The ER is the major Ca²⁺ store of the cell and calreticulin- or Grp94-deficient cells have impaired Ca²⁺ buffering and Ca²⁺ homeostasis (36-43). We examined, therefore, whether ERp57 deficiency had any effect on ER Ca²⁺ homeostasis. Wild-type, ERp57/ERp57ER mouse embryonic fibroblasts were stimulated with thapsigargin, an inhibitor of sarcoplasmic/endoplasmic reticulum Ca²⁺ ATPase (SERCA) type Ca²⁺ pumps or with bradykinin, a potent activator of the inositol 1,4,5-trisphosphate (InsP₃)-dependent Ca²⁺ release channel located in the ER. When cells were stimulated with bradykinin, the peak amplitude and the duration of enhanced cytoplasmic Ca²⁺ concentration was comparable in wild-type, ERp57/ERp57ER cells (Fig. 4A). In ERp57/ERp57ER cells we observed a slight increase in Ca²⁺ concentration in response to thapsigargin when compared to wild-type (Fig. 4B), suggesting that there might be minor changes in ER Ca²⁺ capacity in the absence of ERp57. This might be due to an ERp57-dependent regulation of SERCA2b activity (44).

The Unfolded Protein Response (UPR) in ERp57-deficient cells - Next, we examined if the absence of ERp57 led to any induction of ER stress and consequently the initiation of the UPR. The UPR was examined by splicing analysis of mRNA encoding X-box binding protein (Xbp1), which is cleaved and activated by ER-stress-activated inositol-requiring enzyme (IRE)-1 (2). As a control, ER stress was induced using a classical ER stress inducer, thapsigargin (2). In addition, we induced UPR/ER stress in wild-type and ERp57-deficient cells with brefeldin A (BFA), an ER-Golgi trafficking inhibitor (45).

RT-PCR analysis was carried out to test for expression of Grp78/BiP mRNA and splicing of Xbp1, both markers of UPR. Non-stimulated wild-type and ERp57/ERp57ER cells did not have an increased level of Grp78/BiP mRNA nor was there any observable splicing of Xbp1 mRNA (Fig. 5A). As expected, thapsigargin induced complete splicing of Xbp1 (Fig. 5A, Xbp1s) as well as a significant increase in the level of Grp78/BiP mRNA (Fig. 5A). When wild-type and ERp57/ERp57ER cells were treated with BFA, there was incomplete splicing of Xbp1 with both unspliced (Fig. 5A, Xbp1us) and spliced (Fig. 5A, Xbp1s) forms of Xbp1 mRNA detected. Interestingly, both the level of Grp78/BiP mRNA and the spliced form of Xbp1 were increased in ERp57/ERp57ER compared to wild-type cells (Fig. 5A), indicating that ERp57/ERp57ER cells were more sensitive to BFA-induced ER stress.

Next, we quantified Xbp1 splicing using a luciferase reporter system developed by Kaufman's group (33). In this system, only the spliced form of the Xbp1 is in frame with the luciferase reporter gene, therefore, high luciferase activity reports splicing of Xbp1 and increased ER stress (33). Figure 5B shows that there was a small but significant increase in the UPR in ERp57/ERp57ER cells when compared to wild-type cells (Fig. 5B) and this was not...
rescued by expression of the recombinant ERp57 (Fig. 5B). Thapsigargin treatment significantly enhanced luciferase activity in wild-type, ERp57-/- and ERp57-/- ERp57ER cells (Fig. 5B). There was an 8-fold increase in UPR activation upon treatment with thapsigargin when compared to non-stimulated counterparts (Fig. 5B). Although there was a significant increase in ER stress in the absence of ERp57, this did not result in increased apoptosis in ERp57-/- cells (data not shown) indicating that ERp57-deficient cells experienced a tolerable level of ER stress (46). ERp57 is one of the targets of oxidative stress induced by H2O2 (47), therefore, we also used H2O2 to stress (46). ERp57 is one of the targets of oxidative stress in ERp57-deficient cells. H2O2 was not a very potent inducer of ER stress as there was only a slight increase in ER stress in cells treated with H2O2 (Fig. 5C). We concluded that ERp57 deficiency did not have a significant effect on ER morphology; expression of ER associated chaperones and folding enzymes, ER stress, or apoptosis.

**STAT3 Activation in the Absence of ERp57 -** Earlier reports indicate that ERp57, in addition to its function as an ER oxidoreductase folding enzyme, may play a role in modulation of the STAT3 signalling (6,15,48). It has been proposed that ERp57 forms complexes with both inactive and active STAT3, preventing STAT3 interaction with DNA and consequently leading to inhibition of STAT3-dependent signalling pathway (6,48). Therefore we took advantage of ERp57-deficient cells and examined whether the absence of ERp57 affected STAT3 signalling. STAT3 signalling was monitored using RT-PCR, Western blot analyses of inactive and active (phosphorylated-Y705) STAT3, and luciferase activity assay for STAT3 activated promoter.

Figure 6A shows that expression of STAT3 mRNA was not affected in the absence of ERp57 (ERp57-/-) nor in ERp57-deficient cells transfected with ERp57 expression vector encoding ERp57 localized to the ER (ERp57-/- ERp57ER). Tubulin expression was measured as a control. However, Western blot analysis showed increased levels of STAT3 protein and phospho-STAT3 (Y705) (Fig. 6B). The level of phospho-STAT3 was not changed in ERp57-/- ERp57ER or ERp57-/-ERp57cyt (Fig. 6B). To quantify STAT3 activation we used pLucTKS3 plasmid DNA containing firefly luciferase reporter gene under the control of STAT3 binding sites (34). Since ERp57 has been shown to inhibit STAT3 activity, we expected that STAT3-dependent activation of the promoter would result in high activity of luciferase in the absence of ERp57. Wild-type, ERp57-/-, ERp57-deficient cells expression ER target ERp57 (ERp57-/-ERp57ER), cytoplasm target ERp57 (no N-terminal signal sequence, ERp57-/-ERp57cyt) and green fluorescent protein (GFP) control were transfected with luciferase reporter plasmid followed by analysis of luciferase activity. In non-stimulated ERp57-/- cells, there was 2-fold increase in STAT3 activation (Fig. 6C) indicating that there was a link between ERp57 and STAT3 activity. Since STAT3 is a cytoplasmic/nuclear transcription factor found in the cytoplasm, for ERp57 to interact directly with STAT3 to form functional complexes, ERp57 would have to be also present in the cytoplasm. Therefore, we tested if the ER targeted or cytoplasm targeted ERp57 was effective in modulation of STAT3 signalling. High activity of STAT3 in ERp57-/- cells was partially rescued in ERp57-deficient cells expressing ER targeted ERp57 (Fig. 6C, ERp57-/- ERp57ER) but not in ERp57-/- cells expressing cytoplasm targeted protein (Fig. 6C, ERp57-/-ERp57cyt). As expected, expression of the recombinant GFP in ERp57-deficient cells did not have any effect on STAT3 transcriptional activity. Figure 3D shows that there was a detectable level of immunoreactive ERp57 at the cell surface. However, we do not believe that this small fraction of ERp57 contributes to the modulation of STAT3 transcriptional activity since addition of purified ERp57 to cell cultures did not affect STAT3 transcriptional activity in the ERp57-deficient cells (Supplementary Figure 1). We concluded that the ER targeted ERp57 was the most effective in influencing STAT3 signalling, suggesting that ERp57 modulates STAT3 activity from the lumen of the ER.

**Calreticulin Enhances ERp57 Modulation of STAT3 Signalling -** ERp57 forms functional complexes with calreticulin to assist in folding and posttranslational modification of newly synthesized proteins (3). We asked whether calreticulin may influence ERp57-dependent STAT3 activity. To do this we employed crt-/- cells lines expressing wild-type calreticulin or calreticulin mutants defective in binding ERp57 (32). In this study we used a loss of ERp57 binding calreticulin-E239R mutant and a calreticulin-G242A mutant that has no loss in ERp57 binding (32). Western blot analysis showed that STAT3 or phospho-STAT3 (Y705) expression was not affected by expression of calreticulin or calreticulin mutants in crt-/- cells (Fig. 7). Surprisingly, quantitative analysis of STAT3 activity using luciferase reporter gene system showed high activity of STAT3 in crt-/- cells compared to wild-type counterparts (Fig. 7B), indicating that calreticulin may also affect STAT3 signalling. Figure 7B shows that STAT3 was not affected in the absence of calnexin, a homologue of calreticulin. Expression of wild-type...
calreticulin (Fig. 7B, crt\(^{-/-}\)-CRT) or a calreticulin mutant interacting with ERp57 (Fig. 7B, crt\(^{-/-}\)-CRT\(_{239R}\)) resulted in full recovery of STAT3 activity back to the level observed in wild-type cells (Fig. 7B, wt). In contrast, expression of calreticulin mutant with a loss of ERp57 binding had no effect on STAT3 activity (Fig. 7B, crt\(^{-/-}\)-CRT\(_{239R}\)), which remained high and at the same level as observed in calreticulin deficient cells (Fig. 7B, crt\(^{-/-}\)). Taken together, these results indicate that ERp57-dependent modulation of STAT3 from the lumen of the ER requires calreticulin capable of forming complexes with ERp57.

**DISCUSSION**

In this study, we showed that deletion of the Pdia3 gene, which encodes ERp57, was embryonic lethal at E13.5. Biochemical and cell biological analysis of ERp57-deficient cells indicated that the absence of ERp57 has no impact on ER morphology, expression of ER associated chaperones and folding enzymes, ER stress, or apoptosis. However, we show that STAT3-dependent signalling was increased in the absence of ERp57 and this could be rescued by expression of the ER targeted ERp57, indicating that ERp57 affects STAT3 signalling from the lumen of the ER. This effect of ERp57 on the STAT3 pathway is further enhanced by an ER luminal complex formation between ERp57 and calreticulin. Increased STAT3 activity in the absence of ERp57 may contribute to embryonic lethality of ERp57\(^{-/-}\) mice.

The ERp57 gene is differentially regulated during embryonic development indicating that the protein may play a direct role in the development of specific tissues. This is in line with earlier observations of the expression of ER associated ERp57 partner proteins, calreticulin and calnexin (2,23). Calreticulin is predominately expressed in the heart during early stages of embryonic development and calreticulin-deficient mice die at E14.5 from impaired cardiac development (23). In contrast the calnexin gene is highly activated in neuronal tissue (2) and calnexin-deficient mice are born with neurological abnormalities (49). In this study, we showed that the ERp57 gene was highly active in the inner cell mass of blastocysts, an origin of the fetus formation (50) and in the lung, liver, and brain during early stages of embryogenesis, suggesting that impaired development of these tissues may, at least in part, contribute to ERp57\(^{-/-}\) embryonic lethality. The high expression of ERp57 in the lungs seen in this study is of interest because it has recently been reported that ERp57 is decreased in a neonatal rat model of hyperoxia-induced lung injury and cultured cells (47). siRNA knockdown of ERp57 in human endothelial cells resulted in cellular protection against hyperoxia and tunicamycin-induced caspase-3 activation apoptosis, suggesting that ERp57 has a role in the regulation of apoptosis (47).

Garbi et al. (4) carried out a targeted deletion of the ERp57 gene in mouse B cells. They also reported no ERp57\(^{+/+}\) offspring, indicating that an ERp57 deficiency might be embryonic lethal. Here we investigated embryonic development in the absence of ERp57 and showed that ERp57\(^{-/-}\) was embryonic lethal at E13.5. Interestingly, deletion of ERp57 in B cells in mice results in normal B cell development, proliferation, and antibody production indicating that ERp57 is not required for glycoprotein folding in B cells (4). However, there is aberrant assembly of the peptide loading complex, showing that ERp57 is involved in the assembly of the peptide loading complex and it contributes both qualitatively and quantitatively to MHC class I antigen presentation in vivo. This supports earlier observations that ERp57 is critically involved in the early folding events of MHC class I heavy chain (5,14). The role of ERp57 in MHC class I biogenesis and assembly is further supported by ERp57 siRNA studies (5,51,52).

In addition to its role as a folding enzyme in quality control in the secretory pathway, ERp57 is reported to affect STAT3 signalling (12). This may be due to formation of inhibitory complexes between ERp57 and STAT3 protein either in the cytoplasm or the nucleus (6,15,48,53,54). Furthermore, in avian and mammalian cells, DNA-protein cross-linking experiments indicate that ERp57 is nuclear and it interacts directly with DNA (15). In vitro experiments in HeLa cells showed that ERp57 preferentially binds DNA sequences having characteristic scaffold/matrix associated regions (S/MARs) (55). Finally, the protein has also been suggested to be present on the cell surface and in the cytoplasm (6,14-17). Using FACS analysis we detected some immunoreactive ERp57 on the surface of cells used in this study. However, it is unlikely that the cell surface ERp57 affects STAT3 transcriptional activity as addition of extracellular ERp57 did not affect STAT3 function (Supplementary Figure 1). The conundrum is then: how does ERp57 gain access to the cytoplasm/nucleus to influence STAT3 activity when its N-terminal signal sequence should direct it to the ER and its C-terminal QDEL ER retrieval motif should keep it there? To address this question, we took advantage of available ERp57-deficient cells and tested, for the first time, STAT3 signalling in the absence of ERp57. Analysis of STAT3-dependent expression of the luciferase reporter gene in ERp57-deficient cells revealed that ERp57 indeed affects STAT3 signalling. Most importantly, we showed
that the ER but not the cytoplasmic form of ERp57 was effective in inhibition of STAT3 activity. This effect is specific to ERp57, since transfection of ER targeted GFP or calnexin did not have any effect on the STAT3 activity.

Figure 8 shows a model of ERp57 function in the lumen of the ER. ERp57 is an ER resident folding enzyme involved in oxidative folding of glycoproteins in the ER, biosynthesis of the MHC class I, and as a component of the loading complex (14). In this study, we showed that ERp57 also affects STAT3 signalling from the lumen of the ER (Fig. 8). It is not clear at present how ERp57 affected STAT3 signalling, but our work indicates that the inhibitory function of ERp57 was significantly enhanced by interaction with another ER resident luminal protein, calreticulin (Fig. 8). The two proteins form functional complexes involved in folding and posttranslational modification of newly synthesized (glyco)proteins (42). Our results indicate that ERp57-dependent modulation of STAT3 activity also depends on the formation of ERp57-calreticulin complexes in the lumen of the ER. Interestingly, ERp57-calreticulin complex formation might also be important for cell surface targeting of these ER proteins (56).

The molecular mechanisms of ERp57-dependent signalling from the ER are not presently clear, however, several other cellular processes are controlled by “ER signalling”. For example, cholesterol homeostasis is controlled by ER-nuclear signalling via sterol-regulated proteolysis of ER membrane bound transcription factors called sterol regulatory element-binding proteins (57). The UPR is also regulated by ER associated kinases which transduce their signals from the ER to affect gene expression in the nucleus (58). One critical aspect of Ca²⁺ homeostasis is involved in store-operated Ca²⁺ influx resulting from ER-dependent activation of the plasma membrane Ca²⁺ channel (59). Transcriptional activity of steroid receptors is also modulated by ER luminal proteins (60). Here we report, for the first time, that the STAT3-dependent pathway may also be modulated by signalling from the ER.

REFERENCES


Footnotes

This work was supported by the Canadian Institutes of Health Research (MOP-15291), the Heart and Stroke Foundation of Alberta, and the Alberta Heritage Foundation for Medical Research. Superb technical assistance of Monika Dabrowska and Alison Thorne is greatly appreciated. We thank R. Kaufman and J. Jung for plasmid DNA. H.C. and J.J. are recipients of studentship awards from the Alberta Heritage Foundation for Medical Research. J.G. is supported by the Canadian Institutes of Health Research, Heart and Stroke Foundation of Canada Membrane Protein, and the Cardiovascular Disease Training Program. D.P. was supported by an Alberta Heritage Foundation for Medical Research Summer Studentship and is supported by the Canadian Institutes of Health Research Frederick Banting and Charles Best Canada Graduate Scholarship - Master's Award.

The abbreviations used are: BFA, brefeldin A; E, embryonic day; ER, endoplasmic reticulum; ERp57/−/−ERp57ER, ERp57-deficient cells expressing ER targeted ERp57; ERp57/−/−ERp57cyt, ERp57-deficient cells expressing cytoplasmic targeted ERp57; FACS, fluorescence-activated cell sorting; FITC, fluorescein isothiocyanate; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MHC, major histocompatibility complex; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PDI, protein disulfide isomerase; RT, reverse transcription; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SERCA, sarcoplasmic/endoplasmic reticulum Ca2+ ATPase; STAT, signal transducers and activators of transcription; UPR, unfolded protein responses; Xbp, X-box binding protein.
Legends to the Figures:

FIGURE 1. Disruption of the ERp57 gene and generation of ERp57<sup>−/−</sup> mice.
A. A linear representation of the Pdia3 gene which encodes ERp57. The gene was interrupted by insertion of the pGT1.8TM vector (red). The locations of the F1, F2, and R3 DNA primers used for genotyping are indicated in the figure. Introns are represented as blocks. B. PCR analysis of genomic DNA isolated from wild-type (wt), heterozygote ERp57<sup>+/−</sup> and homozygote ERp57<sup>−/−</sup> embryos. Primers R3 and F1 were used for detection of wild-type alleles and R3 and F2 for mutated allele. C. Western blot analysis with anti-ERp57 antibodies (upper panel). Lower protein band corresponds to a nonspecific immunoreactivity. Anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibodies were used to normalize for protein loading (lower panel). D. Hematoxylin and Eosin staining of embryonic day 12.5 wild-type (wt) and ERp57-deficient embryos (ERp57<sup>−/−</sup>).

FIGURE 2. Activation of the ERp57 promoter during mouse embryogenesis.
Expression of the ERp57 gene was detected as β-galactosidase activity from the β-galactosidase reporter gene inserted in the ERp57 gene within the ERp57<sup>−/−</sup> mouse (see Figure 1). A. Activation of the ERp57 promoter in mouse blastocysts. High activation of the ERp57 gene was detected in the inner cell mass (icm) of the blastocysts but not in the blastocoels (b) or the trophectoderm (t). B. Activation of the ERp57 promoter in lung, liver, and spine but not the heart of E13.5 ERp57<sup>−/−</sup> mouse embryos. C. High activity of the ERp57 promoter in the lung, liver, gut and spinal cord in an E15.5 ERp57<sup>−/−</sup> mouse embryo. D. High activity of the ERp57 promoter in the brain, lungs and liver in an E18.5 an ERp57<sup>−/−</sup> mouse embryo. E. β-galactosidase staining of a wild-type E15.5 control embryo.

FIGURE 3. Western blot and microscopy analysis of ERp57-deficient cells.
A. Western blot analysis of wild-type, ERp57-deficient cells (ERp57<sup>−/−</sup>) and ERp57<sup>−/−</sup> cells expressing full length recombinant ERp57 (ERp57<sup>−/−</sup>-ERp57<sub>ER</sub>) or cytoplasmic-targeted, no signal sequence ERp57 (ERp57<sup>−/−</sup>-ERp57<sub>cyt</sub>). Blots were probed with rabbit anti-ERp57 antibodies (lower protein band corresponds to a nonspecific immunoreactivity) and anti-GAPDH antibodies. In B, Immunostaining of wild-type cells (wt), ERp57-deficient cells (ERp57<sup>−/−</sup>), ERp57-deficient cells expressing full length, ER targeted recombinant ERp57 (ERp57<sup>−/−</sup>-ERp57<sub>ER</sub>), ERp57-deficient cells expressing cytoplasmically targeted recombinant ERp57 (ERp57<sup>−/−</sup>-ERp57<sub>cyt</sub>), calreticulin-deficient cells (crt<sup>−/−</sup>), calreticulin-deficient cells expressing full-length calreticulin (crt<sup>−/−</sup>-CRT), calreticulin deficient cells expression loss of ERp57 binding mutant (crt<sup>−/−</sup>-CRT<sup>E239R</sup>) or ERp57 binding mutant of calreticulin (crt<sup>−/−</sup>-CRT<sup>G242A</sup>). Cells were stained with anti-ERp57 antibodies and with TR-ConA, Texas Red conjugated Concanavalin A. In each panel images represent staining with anti-ERp57 antibodies, ConA staining, phase contrast and merged images of the cells. Scale bar=16 µm. C. Western blot analysis of cell lysate, cytoplasmic and microsomal (containing ER) fraction of wild-type, ERp57-deficient and ERp57-deficient (ERp57<sup>−/−</sup>) cell lines expressing cytoplasmic targeted ERp57 (ERp57<sup>−/−</sup>-ERp57<sub>cyt</sub>). D. Flow cytometry analysis of specific cell lines was carried out with anti-ERp57 antibodies. Wild-type, ERp57<sup>−/−</sup><sub>ER</sub>, ERp57<sup>−/−</sup><sub>Cys</sub>, crt<sup>−/−</sup>, crt<sup>−/−</sup>-CRT, crt<sup>−/−</sup>-CRT<sup>E239R</sup>, crt<sup>−/−</sup>-CRT<sup>G242A</sup> cell lines were used for the analysis. Results are presented as the relative mean florescence intensity after subtracting unspecific staining of ERp57<sup>−/−</sup> cells. M2 represents the gate set on cells stained with antibody. E. Electron microscopy analysis of wild-type and ERp57-deficient cells. The arrows indicate the location of the ER. Scale bar=16 µm. F. Western blot analysis of ER chaperone proteins and oxidoreductases in wild-type (wt) and ERp57-deficient (ERp57<sup>−/−</sup>) mouse embryonic fibroblasts. Antibodies used to probe the Western blot are indicated to the left of each panel. Lower protein band in the ERp57 lanes corresponds to a nonspecific immunoreactivity. CNX, calnexin; CRT, calreticulin; PDI, protein disulfide isomerase.

FIGURE 4. Endoplasmic reticulum Ca<sup>2+</sup> homeostasis in ERp57-deficient cells and cells expressing ER targeted ERp57.
Cytoplasmic Ca<sup>2+</sup> concentration was measured in the wild-type and ERp57-deficient cells as described under “Experimental Procedures”. A. Ca<sup>2+</sup> released by bradykinin. In the absence of ERp57 (white bar) there was a decrease in bradykinin Ca<sup>2+</sup> release but this was not significantly different from wild-type (black bar). Bradykinin release is decreased in cells supplied with ER localized ERp57 (ERp57<sup>−/−</sup>-ERp57<sub>ER</sub>; grey bar) but this was not significantly different from wild-type cells. Data is presented as the mean ± SD, n=4. B. Ca<sup>2+</sup> released by thapsigargin. In the absence of ERp57 (ERp57<sup>−/−</sup>, white bar) and in...
ERp57<sup>−/−</sup> cells transfected with expression vector encoding ERp57 (ERp57<sup>−/−</sup>-ERp57<sub>ERp57</sub> grey bar) showed significantly more Ca<sup>2+</sup> release. Data is presented as the mean ± SD, n=4. Two-sample, unpaired t-test was performed. *p=0.01 vs. wild-type and *p=0.0065 vs. wild-type.

**FIGURE 5. Endoplasmic reticulum stress in the absence of ERp57.**
Endoplasmic reticulum (ER) stress was induced in wild-type and ERp57-deficient cells with thapsigargin (TG; 1 µM) or Brefeldin A (BFA; 2.5 µM). U=untreated. A. RT-PCR analysis of Grp78/BiP mRNA and splicing of Xbp1 mRNA (2). Spliced (Xbp1s) and unspliced (Xbp1u) forms of Xbp are indicated with arrows. RT-PCR of GAPDH mRNA was carried out to normalize for loading of agarose gels. B. Quantitative analysis of splicing of the Xbp1 mRNA in wild-type and ERp57-deficient cells (ERp57<sup>−/−</sup>). Cells were transfected with DNA plasmid reporting Xbp1 splicing. ER stress was induced with 1 µM thapsigargin (TG) or 50 µM H<sub>2</sub>O<sub>2</sub>. Renilla luciferase and firefly luciferase activities were measured and the relative ratio of firefly luciferase to Renilla luciferase activity in each cell lysate is reported. Data are presented as the mean ± SD, n=9. RLU, relative light units. Two-sample, unpaired t-test was performed. *p=0.01 vs. wild-type and *p=0.02 vs. wild-type.

**FIGURE 6. STAT3 expression and activity in the absence of ERp57.**
A. RT-PCR analysis of the STAT3 mRNA in wild-type (wt), calreticulin deletion (crt<sup>−/−</sup>), ERp57<sup>−/−</sup>-ERp57<sub>ERp57</sub> and ERp57<sup>−/−</sup>-ERp57<sub>cyt</sub> cells. B. Western blot analysis of STAT3 (inactive) and phosphorylated (Y<sup>705</sup>) STAT3 (active) in wild-type (wt), ERp57<sup>−/−</sup>-ERp57<sub>ERp57</sub> and ERp57<sup>−/−</sup>-ERp57<sub>cyst</sub> expressing cells. ERp57<sup>−/−</sup>-GFP expressing cells were used as a control. Cells were transfected with a plasmid containing the luciferase reporter gene under control of the STAT3 activated promoter. Renilla luciferase and firefly luciferase activities were measured as described under “Experimental Procedures,” and the relative ratio of firefly luciferase to Renilla luciferase activity in each cell lysate is presented. Data are the mean ± SD. (wild-type, n=27; ERp57<sup>−/−</sup>, n=21, ERp57<sup>−/−</sup>-ERp57<sub>ERp57</sub> n=12; ERp57<sup>−/−</sup>-ERp57<sub>cyst</sub> n=12; and ERp57<sup>−/−</sup>-GFP n=9.) RLU, relative light units. Two-sample, unpaired t-test was performed. *p=0.0013 vs. wild-type and *p=0.0001 vs. wild-type. **p=0.0031 vs. ERp57<sup>−/−</sup>-ERp57<sub>ERp57</sub>.

**FIGURE 7. Calreticulin enhances ERp57 effects on STAT3 activity.**
A. Western blot analysis of STAT3 (inactive) and phosphorylated (Y<sup>705</sup>) STAT3 (active) in wild-type (wt), calreticulin-deficient cells (crt<sup>−/−</sup>), calreticulin deficient cells with loss of ERp57 binding mutant (crt<sup>−/−</sup>-CRT<sub>E239R</sub>) or calreticulin-deficient cells with no loss of ERp57 binding (crt<sup>−/−</sup>-CRT<sub>G242A</sub>). Western blot analysis of tubulin was used as loading control. B. STAT3 activity in wild-type (wt), calreticulin-deficient cells (crt<sup>−/−</sup>), calreticulin deficient cells expression loss of ERp57 binding mutant (crt<sup>−/−</sup>-CRT<sub>E239R</sub>) or ERp57 no loss of binding mutant of calreticulin (crt<sup>−/−</sup>-CRT<sub>G242A</sub>). Renilla luciferase and firefly luciferase activities were measured as described under “Experimental Procedures” and the relative ratio of firefly luciferase to Renilla luciferase activity in each cell lysate is presented. Data are the mean ± SD. (wild-type, n=14; crt<sup>−/−</sup>, n=12, crt<sup>−/−</sup>-CRT<sub>E239R</sub> n=12; crt<sup>−/−</sup>-CRT<sub>G242A</sub> n= 4; and crt<sup>−/−</sup>-CRT<sub>E239R</sub> n=4.) RLU, relative light units. Two-sample, unpaired t-test was performed. *p<0.0001 vs. wild-type and **p=0.02 vs. wild-type. #p=0.0003 vs. crt<sup>−/−</sup>. ##p=0.0088 vs. crt<sup>−/−</sup>.

**FIGURE 8. A model of the relationship between STAT3, ERp57 and calreticulin.**
This model shows cross talk between ERp57, calreticulin and STAT3 signalling. ERp57 forms functional complexes with calnexin and calreticulin to promote protein folding, disulfide bond formation and isomerisation. ERp57 is also critical for MHC class I biosynthesis and assembly. A model shows that ERp57, from the lumen of the ER, also affects STAT3 signalling and functions as a STAT3 inhibitor. ERp57-dependent modulation of STAT3 is enhanced by complex formation between ERp57 and calreticulin.
### Table 1
Genotyping of offspring from ERp57+/− intercross

<table>
<thead>
<tr>
<th>Age of Mouse</th>
<th>Number of Progeny</th>
<th>ERp57 Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+/+</td>
<td>+/−</td>
</tr>
<tr>
<td>E15.5</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>E14.5</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>E13.5</td>
<td>11</td>
<td>3</td>
</tr>
<tr>
<td>E12.5</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>E11.5</td>
<td>15</td>
<td>8</td>
</tr>
<tr>
<td>E10.5</td>
<td>9</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>54</td>
<td>18</td>
</tr>
</tbody>
</table>
Figure 1
Figure 3A

- Wild-type
- ERp57<sup>-/-</sup>
- ERp57<sup>-/-</sup>-ERp57<sub>ER</sub>
- ERp57<sup>-/-</sup>-ERp57<sub>cyt</sub>

GAPDH

ERp57
Figure 3B
**Figure 3B**

* crt*/-

DIC  | ERp57  | ConA  | Merge

* crt*/-CRT

* crt*/-CRT^{E239R}

* crt*/-CRT^{G242A}
Figure 3C
Figure 3D
Figure 3F
**Figure 4**

(A) Calcium Concentration (nM) for wild-type, $ERp57^{-/-}$, and $ERp57^{-/-}$ - ERp57ER.

(B) Calcium Concentration (nM) for wild-type, $ERp57^{-/-}$, and $ERp57^{-/-}$ - ERp57ER.
**Figure 5**

**A**

<table>
<thead>
<tr>
<th></th>
<th>ERp57^{-/-}</th>
<th>wt</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>U</td>
<td>TG</td>
</tr>
<tr>
<td>Grp78/BiP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Xbp1us</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Xbp1s</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**B**

- H$_2$O$_2$ (50 μM)
- TG (1 μM)
- untreated

RLU (Firefly luciferase/Renilla luciferase)

- wild-type
- ERp57^{-/-}
- ERp57^{-/-} - ERp57$_{ER}$
Figure 6
Figure 7
• protein folding
• thiol oxidoreductase
• MHC class I biology

Figure 8
ERp57 modulates STAT3 signalling from the lumen of the endoplasmic reticulum
Helen Coe, Joanna Jung, Jody Groenendyk, Daniel Prins and Marek Michalak

*J. Biol. Chem.* published online December 18, 2009

Access the most updated version of this article at doi: 10.1074/jbc.M109.054015

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at
http://www.jbc.org/content/early/2009/12/18/jbc.M109.054015.full.html#ref-list-1