EXPRESSION OF ENDOPLASMIC RETICULUM OXIDOREDUCTASES (EROS) AND THEIR ROLE IN THE GI TRACT

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CHAPTER 2

MATERIALS AND METHODS

2.1 Antibodies

Monoclonal anti-Ero1α (2G4) was a gift from Prof. Roberto Sitia (Universitá Vita-Salute, Milan, Italy). Anti-Ero1α (D5) was raised in rabbit against reduced, non-reduced and denatured forms of an *E. coli* expressed and purified MBP fusion protein (Mezghrani *et al.*, 2001). Polyclonal rabbit anti-Ero1β (4131) was raised against the unique internal peptide, YTGNAEEDADTKTLL (Dias-Gunasekara *et al.*, 2005). The anti-Ero1β Rabbit Polyclonal 11261-2-AP was purchased from Proteintech. Polyclonal rabbit anti-serum against PDI was raised against purified rat PDI as described (Benham *et al.*, 2000).

The anti-AGR2 antibody, ab22208, was purchased from Abcam, raised against an internal sequence (TQTYEEALYK SKTSNKPL), of Human Anterior Gradient 2 (AGR2). The mouse monoclonal anti-MYC (#2276) was purchased from Cell Signalling.

Polyclonal anti-ERp57 and anti-ERp72 were a gift from Prof. Neil J. Bullied (University of Manchester, UK). Goat Anti-BiP "SC-1050" was purchased from Santa Cruz. Monoclonal anti-HSP90 was from a stock characterised by Takayuki Nemoto and colleagues (K41016; Nemoto *et al.*, 1997) and was a kind gift from Prof. Roy Quinlan (Durham University, UK). Monoclonal HC10 raised against MHC class I heavy chain was characterised by Stam and colleagues (HC10; Stam et al., 1986) and was used as a tissue culture supernatant. For Western blotting, the secondary antibodies used were swine anti-rabbit HRP (SWARPO P0399) and goat anti-mouse HRP (GAMPO P0447) conjugates (commercially available from DAKO). Dilutions used for Western blot are given in Table 2.1. For immunofluorescence work, anti-mouse 488 (A21206) and anti-rabbit 488 (A21202) were purchased from Invitrogen.

Table 2.1 Antibodies used with dilutions for Western blot/Immunofluorescence

Antibody	Details	Dilution for Western blot/ Immunofluorescence
anti-Ero1α	Roberto Sitia: Mouse Monoclonal; "2G4"	1:50
anti-Ero1α	"D5"; Rabbit Polyclonal	1:500
anti-Ero1β	"4131"; Rabbit Polyclonal	1:50
anti-Ero1β	Proteintech "11261-2-AP"; Rabbit Polyclonal	1:50
anti-PDI	Rabbit Polyclonal	1:1000/1:500
anti-AGR2	Abcam ab22208; Rabbit Polyclonal	1:100/1:500
anti-Myc	Cell Signalling #2276: Mouse Monoclonal	1:2000
anti-MHC Class1	Nico Stam: Mouse Monoclonal "HC10"	1:200
anti-ERp57	Neil Bullied: Rabbit Polyclonal #27	1:1000
anti-ERp72	Neil Bullied; Rabbit Polyclonal	1:1000
anti-BiP	Santa Cruz SC-1050: Goat Polyclonal	1:200
anti-HSP90	Takayuki Nemoto: Mouse Monoclonal "41006")	1:500
anti-rabbit HRP	DAKO P0399	1:3000/
anti-swine HRP	DAKO P0447	1:3000
donkey anti-mouse	Fluorescein label (FITC): Immunofluorescence only	1:50
swine anti-rabbit	Rhodamine label (TRITC): Immunofluorescence only	1:50

2.2 Molecular Biology

2.2.1 DNA Constructs used

DNA constructs were used for cellular transfection and DNA purification work. The constructs used for transfection were pcDNA3.1human Ero1α-myc, pcDNA3.1 human Ero1β-myc and pcDNA3.1 human Ero1β-HA. These constructs have been previously described (Benham *et al.*, 2000; Dias-Gunasekara *et al*., 2005). The construct used for the purification of recombinant Ero1β was PGEX human Ero1β-HIS-GST, and was a kind gift of Prof. Neil J. Bulleid (University of Manchester, UK).

2.2.1.1 Primers

Oligonucleotide primers were designed using NCBI Primer Blast (www.ncbi.nlm.nih.gov/tools/primer-blast/), which designs primers according to the standard conventions of primer length (17-28 bp length), GC content (40-60%), and checks them against the NCBI BLAST databases for potential cross priming with other DNA sequences. The top results were then subjected to manual verification against the bioinformatic database, NCBI BLAST: (www.ncbi.nlm.nih.gov/BLAST/). The forward and reverse sequences for commonly used primers are given below in Table 2.2. During experiments to establish which, if any, of the putative three alternate splice variants of Ero1β were expressed, unique primers were designed in order to distinguish the three variants. For a full account of this and details of these primers, see Chapter 4.

Table 2.2 Sequences of common primers

	Forward	Reverse	Product Size (BP)
Ero1α	TGTCCTTTCTGGAATGACATC	AAGATCTTCAGAGCAGTGCC	**991**
Ero1β	CTTTCTGGGCAGAAGATGGC	TTTTGTCACCTGCAAACATGG	**864**
BiP	AGAGCTGTGCAGAAACTCCGGC	CCTCTTCACCAGTTGGGGGAGG	**823**
Actin	CCACACCTTCTACAATGAGC	ACTCCTGCTTGCTGATCCAC	**1089**

2.2.2 Purification of plasmid DNA

DNA for transfection experiments was produced using the mini/maxiprep kit (Qiagen). As per the manufacturer's protocol, transformed DH5α bacterial cells were alkaline-lysed, and processed. Plasmid DNA was eluted in a high salt buffer, and concentrated and desalted by isopropanol precipitation.

For transformation, competent DH5α bacteria were taken from -80 °C storage, and thawed on ice. Per 50 µl of DH5α, 2 ng of stock DNA were added, and gently mixed in an eppendorf tube. This was placed on ice for 10 minutes, pulsed for 90 seconds at 42 °C in a water bath, and returned to ice for 10-15 minutes. To this, 700 µl of LB (per litre; 10 g tryptone, 5 g yeast extract, 10 g NaCl [pH 7]) was added, and then placed within a shaker-incubator for 30 minutes at 37 °C. The tubes were then spun at 1000 g in a minicentrifuge (Eppendorf) for 3 minutes; the supernatant was then removed, and the pellet re-suspended in 100 µl LB. The bacteria were plated out onto an LB-Ampicillin (ampicillin 100 µg ml^{-1}) agar plate, pre-warmed at 37 °C, and grown overnight. The following day, a suitable colony was used to inoculate 2 ml of LB in a sterile 15 ml falcon tube, and the starter culture expanded for 6 hours in a 37 °C shaker-incubator. From this, 200 µl of starter culture was added to 100 ml of sterile LB broth

within a conical flask (a 1:500 dilution), with the culture expanded overnight in a 37 °C radial shaker-incubator.

The 100 ml overnight culture was then transferred to two 50 ml falcon tubes, and pelleted at 6000 g for 15 minutes (Sigma-Aldrich, UK centrifuge). One pellet was then re-suspended in 10 ml of buffer P1 (50 mM Tris-Cl [pH 8], 10 mM EDTA, 100 μg ml^{-1} RNase A), and this re-suspension was used in turn to re-suspend the other pellet. To this, 10 ml buffer P2 (200 mM NaOH, 1% SDS [w/v]) was added, and the tube was inverted 4-6 times to mix, and then incubated on ice for 20 minutes and then spun at 6000 g for 30 minutes, and any solid fragments in the resulting supernatant were removed. The supernatant was then transferred to another sterile 50 ml tube, and spun at 6000 g for 15 minutes (4 °C). The plasmid DNA was then collected by decanting the supernatant to a QiaTip 500 which was pre-equilibrated with 10 ml of buffer QBT (equilibration buffer; 750 mM NaCl, 50 mM MOPS [pH 7], 15% isopropanol [v/v], 0.15% Triton X-100 [v/v]). The QiaTip 500 was then given two 30 ml washes with buffer QC (wash buffer; 1 M NaCl, 50 mM MOPS [pH 7], 15% isopropanol [v/v]), prior to DNA elution with 15 ml buffer QF (elution buffer; 1.25 M NaCl, 50 mM Tris-Cl [pH 8.5], 15% isopropanol [v/v]). The eluate was collected in a 50 ml falcon tube. The DNA was precipitated out of solution by adding 10.5 ml of isopropanol at room temperature, mixed by shaking, and the spun at 15,000 g (4 °C) in a centrifuge for 30 minutes. Following the decantation of the supernatant, the resultant DNA pellet was washed with 5 ml of ethanol and then spun in a centrifuge for 10 minutes at 15,000 g (4 °C). The pellet was then air-dried for 10-15 minutes. Buffer TE (10 mM Tris-Cl [pH 8], 1 mM EDTA) was used to dissolve the DNA pellet in a final volume of 100 μl.

2.2.3 Calculation of DNA concentration/yield

DNA concentration was calculated with a UV spectrophotometer. For the blank recording, 75 µl buffer TE or water was added to an UVette (220-1600 nm range; Eppendorf). To measure DNA concentration, 75 µl of a 1:500 dilution of DNA stock was measured. Absorbencies were read at 260 nm and concentrations calculated for dsDNA (1 OD = 50 µg ml^{-1}), which were then corrected to take into account the 1:500 stock dilution. From this stock, 1 µg $µl^{-1}$ stock solutions were created for use in other experiments such as transfection.

2.2.4 Reverse-Transcriptase PCR

Reverse-Transcriptase PCR (RT-PCR) was used to analyse protein expression in cell culture experiments at the level of the mRNA message. Cells were lysed and processed using 200 µl Tri-Reagent (Tri-reagent protocol was adjusted for 6cm dishes; Sigma-Aldrich T9424). Following lysis, cells were harvested and centrifuged (12,000 g for 10 minutes, 4 °C) to remove cell debris. The aqueous phase was transferred to a fresh centrifuge tube and was stood for 5 minutes at room temperature so that all nucleoprotein complexes would dissociate. Phenol-chloroform (Fisher) was added, the sample was shaken for 15 seconds, allowed to stand for 10 minutes at room temperature, and centrifuged again (12,000 g for 15 minutes, 4 °C). Cellular proteins, DNA and RNA were separated into a red organic phase, an interphase and a colourless upper aqueous phase. The RNA was removed and 100 µl isopropanol was added and the mixture was allowed to stand for 10 minutes at room temperature. This was further centrifuged (12,000 g for 10 minutes, 4 °C). The resulting RNA pellet was isolated by removing the supernatant, washed with 75% ethanol and centrifuged (7,700 g for 5

minutes, 4 °C). Following removal of the ethanol wash, the pellet was air-dried for 5 minutes, before being dissolved in 13 µl of nuclease-free water.

For the Reverse Transcriptase PCR (RT-PCR), the Promega AccessQuick system was used. The composition of the RT-PCR used 25 µl of 2x AccessQuick Master Mix, 1 µg RNA template, and a 1 µM final concentration each of up and downstream primers. The final reaction volume was made up to 50 µl with nuclease free water. The reaction tubes were incubated at 45 °C for 45 minutes to initiate reverse transcription, and then cDNAs were cycled for 40 cycles, following a 2 minute initial denaturation step at 95 °C. Temperatures for specific primers and their validation are given in section 4.2.1, including details of thermal optimisation.

2.2.5 Agarose gel electrophoresis

DNA constructs, digests or RT-PCR products were analysed on 1% agarose gel (1.2 g agarose [electrophoresis grade], 120 ml 1x TAE buffer, with 5.5 µl ethidium bromide) and run at 100 V in 1x TAE buffer. A 1Kb nucleotide marker was used (New England Biolabs).

2.3 Cell Culture

HT1080 Human fibrosarcoma cells were cultured in Dulbecco's Modification of Eagle's Medium (DMEM; Invitrogen), HeLa cells were cultured in Minimum Essential Medium (MEM; Invitrogen) and the Human Oesophageal cells (OE19, OE21, OE33; purchased from ECAC) were cultured in RPMI 1640 (Invitrogen), at 37 °C 5% CO_2. Media were supplemented to give final concentrations of 8% foetal calf serum (FCS; Sigma-Aldrich, UK-Aldrich), 2 mM glutamax (Invitrogen), and 100 µg ml^{-1} Penicillin/Streptomycin (Invitrogen). All cells cultured were adherent, and maintained

in 10 cm diameter dishes with 10 ml of media. Cells were passaged at ~80% confluence; the monolayer was digested with 1% porcine trypsin-EDTA (Invitrogen). The HT1080 cells were passaged at a ratio of 1:10; the OE21 and OE33 cells were passaged at a ratio of 1:4. Treatments were carried out at when the cells reached ~70% confluence, in 6 cm diameter dishes (TPP), in a volume of 4 ml total media, unless stated otherwise.

Cell stocks were maintained by regularly freezing down in FCS with 10% DMSO, at low passage numbers.

2.3.1 Cellular Transfection

Transfection with either lipofectamine 2000 (Invitrogen) or GeneJuice (Novagen) was performed according to manufacturer's instructions for 6 cm diameter dishes, under sterile conditions within a flow hood.

For transfection with lipofectamine 2000, 2.5 µl of lipofectamine was added to 1 ml of Opti-MEM (Invitrogen) in a transfection tube, and allowed to mix for 5 minutes. In addition, 1 µg of DNA was added to another vial of 1 ml of Opti-MEM, and combined with the preceding lipofectamine-OptiMEM mix. This final preparation was allowed to stand for 20 minutes, during which time the cells to be treated were washed with 2 ml of Hank's balanced salt solution (HBSS; Invitrogen), and then with 2 ml of Opti-MEM. The DNA-lipofectamine-OptiMEM mix was then added to the cell monolayer, and returned to the 5% CO_2, 37 °C incubator for 6 hours. Next, the DNA-lipofectamine-OptiMEM mix was removed, and the cells were washed with 1 ml of OptiMEM, then 2 ml of HBSS. Complete growth media was then added to the cells, which were returned to the 5% CO_2, 37 °C incubator overnight. Cell lysates were then harvested the morning of the following day, on ice-cooled dishes with MNT lysis buffer (20 mM MES, 30 mM Tris, 100 mM NaCl, pH 7.4) with 1% Triton X-100 (Sigma-Aldrich, UK),

supplemented with 10 µg ml^{-1} each of the protease inhibitors chymostatin, leupeptin, antipain and pepstatin A, (CLAP) and 20 mM N-ethylmaleimide (NEM) or iodoacetic acid, where appropriate.

For transfection with GeneJuice, 15 µl of GeneJuice were added to 250 µl of OptiMEM, in a transfection tube, and allowed to stand for 5 minutes at room temperature. At the end of the incubation, 5 µg of DNA was added to the GeneJuice-OptiMEM mix, and allowed to rest for 15 minutes at room temperature. Following incubation, the GeneJuice-DNA mix was added directly to 4 ml of fresh culture medium, mixed, and then directly applied to washed cells at ~70% confluency. The cells were then returned to the incubator for 6 hours (37 °C). At the end of the transfection reactions, the cells were washed once with 2 ml of HBSS, and then allowed to grow overnight in normal media (37 °C). Cell lysates were then harvested the following morning with MNT lysis buffer, on ice-cooled dishes.

2.3.1.1 Transfection of oesophageal cell lines

A modification of the above procedure was used to transfect the oesophageal cell lines OE21 and OE33 (OEs), which transfected inefficiently using the method described in 2.3.1. Instead, OE cells were grown until around 70% confluency. The cells were trypsinised, before being re-suspended in a volume of 3 ml RPMI medium. This 3 ml was re-suspended and transferred to a fresh 6 cm dish. The cells were allowed to adhere for a period of 3 hours, which had been established as optimal for obtaining adherent but rounded cells. This procedure ensured suitable cell confluence for transfection, with the OE cells extant in distinct groups or small colonies, as opposed to a continuous cellular monolayer.

2.3.2 Assessment of cell viability

2.3.2.1 Trypan blue assay

The trypan blue assay stains nuclei, and is a standard test of cell viability. For digestion of the cellular monolayer on a 6 cm dish, 250 µl of 1% trypsin-EDTA was added, the dish was tilted to cover the surface area, and the excess trypsin aspirated. Once the cells were detached, they were re-suspended in 4 ml PBS. From this, 10 µl was taken to an eppendorf tube, and mixed with 10 µl of 0.4% trypan blue (Sigma-Aldrich, UK-Aldrich). For counting, 10 µl of the DPBS/cells/trypan blue mix was added to a haemocytometer, and the number of single stained (blue) and unstained cells were counted within the central grid. The number of viable cells was calculated as a percentage, upon counting the number of cells per ml on the haemocytometer grid. Following pH treatment (see Chapter 3), cells treated at low pH 1-4 would not trypsinise for a standard cell count, so the cells were stained in situ with 250 µl of trypan blue, and imaged using an inverted microscope with a digital camera, to give an estimate of viability within the field of view.

2.3.2.2 Crystal violet assay

The crystal violet assay was also used for cell staining (method taken from Yamamoto et al., 2007). Following washing in DPBS, 1 ml of 0.2% crystal violet solution (Sigma-Aldrich, UK-Aldrich) in 2% ethanol was applied to cells in a 10 cm dish, and placed on a gentle tilting shaker for 10 minutes a room temperature. The crystal violet was then aspirated, and the cells were washed three times with distilled water. At this step, the cells could either be photographed on the inverted microscope following the addition of

2 ml DPBS to prevent desiccation, or solubilised with 1% SDS, to be analysed by a spectrophotometer at a 1 in 1000 dilution at OD 600.

2.3.3 Preparation of cell culture medium for treatment

Stock solutions of diamide, sodium deoxycholate and sodium chenodeoxycholate (Sigma-Aldrich, UK) were prepared in advance by dissolving the stock solid in water, as per company directions. These were then aliquoted and stored at -20 °C. Immediately prior to treatment, the appropriate volume of reagent was added to an aliquot of sterile media, to give the required final concentration (Jenkins *et al.*, 2004, Jenkins *et al.*, 2008).

2.3.4 Cell culture media pH range treatments

The stock serum-free media bottles were warmed to 37 °C, wiped with 70% ethanol, and opened within the cell culture hood. Aliquots were taken and stored in a 50 ml falcon tube. These were pH-adjusted by adding either HCl or NaOH dropwise to the media (Jenkins *et al.*, 2004, Jenkins *et al.*, 2008). The pH was measured using a calibrated pH meter and checked using pH paper (VWR). These pH-adjusted aliquots were sterilised with syringe filter drive units (Millipore) within the cell culture hood, and then kept at 4 °C until needed.

2.3.5 Cell lysis

Cells were lysed with 200-300 µl of MNT lysis buffer. The lysed cells were scraped, and the lysates were collected into eppendorf tubes. These were centrifuged at 16100 g

(Eppendorf microcentrifuge) for 10 minutes at 4 °C to remove nuclei. The supernatants were then collected into fresh eppendorf tubes and snap frozen in liquid nitrogen.

2.3.6 Immunofluorescence

In order to verify the localisation of proteins of interest, immunofluorescent cell staining was used, on cells that had been seeded direct to sterile glass cover slips.

2.3.6.1 Seeding to coverslips

Circular 13 mm diameter, glass cover slips (VWR) were prepared by coating them with a 0.01% (w/v) polylysine solution, according to manufacturer's instructions (Sigma-Aldrich). They were then sterilised by washing them in 95% ethanol. These sterile cover slips were then added to cell culture dishes.

Following trypsinisation and re-suspension, cells were seeded onto 6 cm dishes containing polylysine cover slips described above. They were allowed to adhere overnight, ready for experimentation the following day.

The cells were washed three times with PBS ++ (Invitrogen/Gibco PBS supplemented with 0.5 mM $MgCl_2$ and 1 mM $CaCl_2$). The cells were fixed onto cover slips with 4% paraformaldehyde for 10 minutes. Following a further three washes in PBS ++, free aldehyde groups were quenched with 50 mM NH_4Cl for 15 minutes (this decreases auto-fluorescence due to paraformaldehyde). Cells were premeabilised with ice-cold methanol for 10 minutes, then were given a further three PBS ++ washes (supplemented with 0.2% BSA). The cover slips were incubated facedown in 25 µl of primary antibody for 30 minutes in a moist chamber, at room temperature (antibody prepared in PBS ++ [0.2% BSA] and centrifuged). The cover slips were washed three times in PBS ++, before facedown incubation in the appropriate secondary antibody for

30 minutes (prepared as primary, above; anti-mouse 488 Alexafluor A21206, anti-rabbit 488 Alexafluor A21202). After a final three washes in PBS ++, the cover slips were mounted on microscope slides with Vectashield, fixed in position with nail varnish (Rimmel, London), and allowed to dry overnight in darkness. Staining was visualised using an inverted Carl Zeiss UV fluorescence microscope (Axiovert 200M), using the Axiocam and associated Axiovision 4.8 software.

The ER or lysosomes were visualised using ER tracker or Lyso tracker stains (Invitrogen). These were added at a 1:1000 dilution directly to the medium for 30 minutes.

2.3.7 Sectioning of cell culture pellets

Cell culture pellets were prepared by trypsinising adherent cells from three 10 cm dishes. These were spun for 5 minutes at 1,500 g and washed twice by re-suspending in PBS. The cell suspensions were transferred to 1.5 ml eppendorf tubes, and spun again in a microcentrifuge at 10000 g for 10 minutes, at 4 °C. They were re-suspended in 4% (w/v) paraformaldehyde, and spun again with the excess paraformaldehyde taken off. The pellets were then snap-frozen in liquid nitrogen, and embedded in tissue-tek (Agar scientific). Cell pellets were sectioned to 5 μm thickness on a Leica cryostat (Model: CM1850UV).

2.4 SDS-PAGE

Cell lysates were analysed by both reducing and non-reducing SDS-PAGE; non-reducing SDS-PAGE can be used to examine oxidation states of a protein, if applicable. For each non-reduced sample to be loaded, 15 μg protein was added to 15μl of 2x Laemmli sample buffer (Laemmli, 1970; 1M Tris [pH 6.8], 20% SDS, 5% glycerol, 0.01% bromophemol blue). In order to reduce the disulphide bonds within the proteins

of interest, 1.5 µl of 500 mM dithiothreitol (DTT) was added per 15 µl sample (to give a final concentration 50 mM). Prior to loading, samples were boiled at 95 ^{o}C for 5 min to denature the proteins, and spun for 15 seconds at 5000 g to return condensed liquid to the bottom of the tube. The samples were then analysed by SDS-PAGE. Gels were cast using the Amersham/Hoefer multi gel casting system. All-blue prestained protein standards (Bio-Rad) were used to determine relative molecular weight of detected proteins. Typically, for detecting proteins above 25 KDa, the running/resolving gel consisted of 8% acrylamide, 0.375 M Tris (pH 8.8) and 0.1% SDS, 0.1% APS, 0.04% N,N,N',N'-Tetramethylethylenediamine (TEMED). In order to detect smaller molecular weight proteins, the running gel was adjusted to 10% acrylamide. The stacking gel consisted of 5% acrylamide, 0.125 M Tris (pH 6.8), 0.1% SDS, 0.075% APS and 0.075% TEMED.

2.5 Western blotting

Following SDS-PAGE, proteins were transferred from the gel to methanol-activated PVDF membranes (Millipore) in transfer buffer (190 mM glycine, 25 mM Tris in 20% Methanol). Transfer was completed using a Bio-Rad transfer apparatus at 150 mA for 2 hours, or 30 V overnight in a fridge at 4 °C. Following transfer, membranes were blocked for 1 hour in an 8% milk solution in TBST (10 mM Tris [pH 8], 150 mM NaCl, 0.05% Tween-20) on a shaker, at room temperature. Membranes were transferred to 50 ml tubes, and rolled whilst being exposed to a primary antibody in 1 ml of 5% milk for 1 hour at room temperature. A list of primary antibody dilutions and species is given in Table 2.1. Following incubation with primary antibody, the membrane was washed three times with 10 ml TBST for 5 minutes. The membrane was then incubated in secondary antibody (anti-mouse or anti-rabbit; 1:3000 [DAKO]) for 1 hour in 5% milk at room temperature, and then washed three times as before in TBST, before being

touch-dried with tissue, and placed onto enhanced chemi-luminescence reagents 1 and 2 (ECL; Amersham Biosciences) for 1 minute. Membranes had excess ECL blotted off onto tissue, before being exposed to Kodak Bio-Max film in dark room conditions. Films were developed using a compact X4 developer machine.

Densitometry was unavailable to quantify protein signal on Western blots. Protein levels were standardised as described above, and the linearity of the resultant ECL signal was judged by multiple exposures of the blotting membranes: 10 seconds, 30 seconds, 1 minute, 2 minutes, 5 minutes, and overnight exposure.

2.5.1 Screening monoclonal antibodies

Putative monoclonal antibodies for Ero1α and Ero1β were generated against purified fusion proteins (see Chapter 5 for full details).

To establish if these monoclonal antibodies were cross-reactive with positive tissues, the following screening strategy was adopted. As Ero1β is highly expressed in the pancreas and stomach (mRNA and immunohistochemical analysis of human and murine tissue (Pagani *et al*., 2000, Dias-Gunasekara *et al*., 2005), murine pancreas and stomach were used as positive controls, as well as Ero1β-myc transfectant lysate. Ero1α-myc transfectants were used as negative controls for Ero1β, which were positive for Ero1α. In re-testing potentially positive monoclonal supernatants, the screening panel was expanded to include rat pancreatic and liver tissue, and a sample of the Ero1β-HIS fusion protein used as the primary immunogen.

2.5.1.1 Lysis of animal tissues

Animal tissue samples were obtained from CD1 mice, and rats (mixed sex). Heart, stomach, liver and pancreas were dissected from the animal within 30 minutes after death. Organs were washed with PBS; excess PBS was drained using tissue paper. The

organs were then snap frozen in liquid nitrogen, and crushed using a mortar and pestle. The tissue was weighed and lysed with 400 µl MNT per 100 mg of tissue. The samples were then taken up in Laemmli buffer as described above (2.3.5). The Biorad DC protein assay (see below) was used to determine protein concentration and cell samples were matched accordingly. Mouse/rat tissues were loaded for SDS-PAGE as described (See 2.4).

2.5.2 Determination of protein concentration

In order to match cell and tissue lysates, the Bio-Rad DC protein assay was used. This assay was based on an original protocol by Lowry (Lowry *et al.*, 1951), whereby protein is reacted with alkaline copper tartrate solution and folin reagent over 15 minutes. Samples and standards were prepared according to the manufacturer's instructions, and scaled down by a factor of five. Five 20 µl standards (0.25, 0.5, 1, 1.5, 2, 2.5 mg ml^{-1}) were used with the appropriate reagents (20 µl reagent S added per 1 ml of reagent A to make regent A'; 20 µl protein sample/standard + 100 µl reagent A' + 800 µl reagent B). The resultant blue colour was measured at an absorbance of 750 nm. The lysis buffer MNT without the alkylating agent NEM or protease inhibitor cocktail, CLAP, was used as the blank, and for diluting BSA standards.

2.6 Protein gel staining

In order to assess proteins on an 8% SDS-PAGE gel, the SimplyBlue SafeStain was used according to manufacturer's instructions. It has a detection limit of 5-10 ng. Following electrophoresis, the gel was removed from the plates and placed into a microwaveable container with 100 ml of distilled water, and microwaved at the 'high'

setting (900-1100 Watts) for 1 minute. The container was checked to ensure the solutions did not boil. The protein gel was then shaken for 1 minute, the water was discarded, replaced with another 100 ml and the microwave/shaking process was repeated a further two times. After removal of the water at the final shaking step, 20 ml of SimplyBlue SafeStain was added to the container, which was microwaved a final time for 1 minute. The container was shook for 5 minutes, and then the staining solution was removed before washing the gel with 100 ml distilled water for 10 minutes. The stained gel was then dried to a piece of filter paper (Whatman), using a vacuum gel drier.

2.6.1 Protein Gel staining (Alternate Method)

For protein purification, an alternate staining method was used. Coomassie blue stain was prepared by making a solution comprising 2 g L^{-1} of brilliant blue powder (Sigma-Aldrich, UK-Aldrich), 50% ethanol and 7.5% acetic acid in dH_2O. The final solution was then gravity filtered using filter paper (Whatman).

Protein gels were fixed for 10-15 minutes in 10% acetic acid, 1% methanol (in dH_2O), on a rocking incubator. The gels were then stained in coomassie blue solution for at least one hour and destained in 50% (v/v) methanol in water with 10% (v/v) acetic acid.

2.6.2 Silver Staining

Silver staining of protein gels (adapted from Morrissey, 1981) was used for the gel based Ero1β assay (Chapter 4). SDS-PAGE gels were transferred to a suitable container and washed briefly with dH2O. They were then washed with 50% methanol for 10 minutes, and washed with water for a further 10 minutes. The washed gel was then incubated with 0.02% sodium thiosulphate solution for 1 minute and rinsed twice with dH_2O. The gel was pre-stained with 0.1% silver nitrate for 20 minutes at 4 °C and

rinsed twice with water. The gel was developed for a few minutes or longer as required using 0.04% formalin, 2% potassium carbonate (in dH_2O) assisted by gentle shaking. The developing reaction was stopped using 5% acetic acid. The gels were then rinsed in water, and scanned using a flatbed scanner, and dried using a gel dryer if necessary.

2.7 Protein Purification, Mutagenesis, and functional assays

2.7.1 Ero1L-B-HIS-GST Purification

A small scrape from the Ero1L-B HIS GST expressing origami cells (Bulleid lab, stock ID: pgex4t-3 glyc 18/09/07) was used to inoculate 5 ml of sterile LB (10 g Tryptone, 5 g Yeast, 10 g NaCl, per litre of milliQ water), with final concentrations of the following antibiotics: 100 µg ml^{-1} Ampicillin, 30 µg ml^{-1} Chloramphenicol, 10 µg ml^{-1} Tetracycline and 10 µg ml^{-1} Kanamycin. This was cultured overnight in a shaker incubator at 37.5 °C.

The following day, the 5 ml starter culture was used to inoculate 500 ml of sterile LB containing 100 µg ml^{-1} Ampicillin, 30 µg ml^{-1} Chloramphenicol, 10 µg ml^{-1} Tetracycline and 10 µg ml^{-1} Kanamycin. This was cultured for 6 hours at 37.5 °C.

At the end of this growth period, the culture was prepared for induction. To the existent 500 ml culture, another 100 ml LB, plus antibiotics at the same concentration described above, was added to accommodate further bacterial growth in the culture. The culture was induced with 500 µM Isopropyl β-D-I-thiogalactopyranoside (IPTG) and 10 mM FAD for 24 hours at 16 °C to induce protein expression.

The next day, the culture was spun in 1 litre centrifuge bottles at 6238 g for 10 minutes. The culture pellet was re-suspended in 40 ml ice-cold 1xPBS, and collected in a 50 ml falcon tube, which was then spun at 6238 g to re-generate the pellet. The PBS was decanted, and the pellet was then snap-frozen in liquid nitrogen and stored at -80 °C.

Bacterial pellets were lysed in 20 ml lysis buffer (20 µg ml^{-1} DNAse [Sigma-Aldrich, UK] 2.5 µgml^{-1} RNAse A [Sigma-Aldrich, UK], 2x EDTA-free protease inhibitor tablets [Roche], 1 mM EDTA, 0.1% Triton X-100 [Sigma-Aldrich, UK], 10 mM Imidazole [Sigma-Aldrich, UK] in 1x PBS). The lysates were then snap-frozen in liquid nitrogen and thawed in a 37 °C waterbath. This was done three times to aid solubilisation.

The lysate was clarified at 14000 g for 10 minutes. The soluble fraction was then incubated with 1ml of nickel agarose (Ni-NTA beads; Qiagen) for 90 minutes, in a 20 ml chromatography column (Bio-Rad). The unbound fraction was drained, and the remaining Ni-NTA beads bound to Ero1β-HIS-GST were then washed with 5 ml wash buffer 1 (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM Imidazole, 0.1% Tween-20, 10% glycerol, pH 8) and wash buffer 2 (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM Imidazole, 0.1% Tween-20, pH 8) before being eluted in 2.5 ml of Ni-NTA elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM Imidazole, 0.1% Tween-20, pH 8). This Ero1β-HIS-GST eluate was then buffer exchanged into 3.5 ml 1x PBS using a PD10 column (GE Healthcare).

The following day, the 3.5 ml Ero1β-HIS-GST sample in PBS was incubated with glutathione sepharose beads for 4 hours. Following this, thrombin was added (100 units ml^{-1}) for 3 hours. The solutions were then gravity filtered in a chromatography column. The flow though contained Ero1β-HIS, while the GST tag remained attached to the beads.

The 3.5 ml Ero1β-HIS flow through was incubated with 500 µl Ni-NTA beads for 90 minutes. The unbound fraction was allowed to drain through the chromatography column, and the Ni-NTA beads were washed and eluted as before, in 2.5 ml Ni-NTA elution buffer. This was then buffer exchanged on a PD10 column into 3.5 ml 50 mM tris, leaving an eluate containing Ero1β-HIS.

Protein concentration was calculated by taking spectrophotometer readings at 280 nm, and at 454 nm, a measure of absorbance of both protein and FAD.

For Ero1β, this was as follows:

$$\frac{\text{280 nm reading}}{\text{MW of Protein}} * 1{,}000{,}000 = X\ \mu M$$

Eluate Volume (ml) * (X μM/10) = X mg ml^{-1}

2.7.2 PDI-HIS and TRX-HIS Purification

A small scrape from the PDI-HIS expressing BL21 cells (Bulleid lab) was used to inoculate 5 ml of sterile LB (10 g Tryptone, 5 g Yeast, 10 g NaCl, per litre of milliQ water), with a final concentration of 100 μgml^{-1}ampicillin. This was cultured overnight in a shaker incubator at 37.5°C.

The following day, the 5 ml starter culture was used to inoculate 500 ml of sterile LB containing 100 μg ml^{-1} ampicillin. This was cultured at 37.5 °C until an OD 600 of 0.5 was reached. At the end of this growth period, the culture was induced for 3 hours with 500 μM IPTG.

For the TRX, a small scrape from the TRX-HIS expressing BL21 cells (Bulleid lab) was used to inoculate 5 ml of sterile LB (10 g Tryptone, 5 g Yeast, 10 g NaCl, per litre of milliQ water), with a final concentration of kanamycin at 10 μgml^{-1}. This was cultured overnight in a shaker incubator at 37.5 °C.

The following day, the 5 ml starter culture was used to inoculate 500 ml of sterile LB containing 10 μg ml^{-1} Kanamycin. This was cultured at 37.5 °C, until an OD 600 of 0.5 was reached. At the end of this growth period, the culture was induced for 3 hours with 500 μM IPTG.

For both cultures the following day, they were spun in 1 litre centrifuge bottles at 14000 g for 10 minutes. The culture pellet was re-suspended in 40 ml ice-cold 1xPBS, and collected in a 50 ml falcon tube, which was then spun again at 3000 g. The PBS was decanted, and the pellet was then snap-frozen in liquid nitrogen, and stored at -80 °C.

Bacterial pellets were lysed in 20 ml lysis buffer (20 µg ml^{-1} DNAse 1, 2.5µg ml^{-1} RNAse A, 2x EDTA-free protein inhibitor tablets (Roche Diagnostics), 1 mM EDTA, 0.1% Triton X-100, 10 mM Imidazole in 1x PBS).

The pellets were then snap-frozen in liquid nitrogen and thawed in a 37 °C waterbath. The lysate was clarified at 12700 g for 10 minutes. To the soluble fraction, 1% Triton X-100 was added (from a 10% stock). The soluble fraction was then incubated with 1 ml of nickel agarose (Ni-NTA beads; Qiagen) for 90 minutes. The Ni-NTA beads left in the chromatography column, bound to Ero1β-HIS-GST, were then washed with 5 ml wash buffer 1 (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM Imidazole, 0.1% Tween-20, 10% glycerol, pH 8) and wash buffer 2 (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM Imidazole, 0.1% Tween-20, pH 8) before being eluted in 2.5 ml of Ni-NTA elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM Imidazole, 0.1% Tween-20, pH 8). This PDI/TRX eluate was then buffer exchanged into 3.5 ml 1x PBS using a PD10 column (GE Healthcare).

2.7.3 Centrifugal concentrating of protein solutions

In order to remove excess buffer from protein containing solutions, the vivaspin method was used (Sartorius). This was useful for concentrating a 3.5 ml elution to a smaller volume of 500 µl. The buffered protein solution was added to the upper compartment of the vivaspin tube, and centrifuged at 3000 g for 10 minutes, repeatedly,

until the correct final volume was reached. At the end of each cycle, the upper compartment liquid was mixed gently with a pipette, as this helped prevent the protein from precipitating out of solution.

2.7.4 Oxygen electrode recordings

Oxygen consumption was measured using a Clark-type oxygen electrode (Rank Brothers, Cambridge, UK). Foil was used to cover the chamber to prevent photoreduction of flavin (FAD). Assays with PDI were carried out in 500 µl using 100 µM reduced PDI and 2 µM Ero1β-HIS in 50 mM Tris–HCl buffer, pH 7.5 with or without 10 mM GSH (Baker *et al.*, 2008).