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## **Intracellular Catalysis of Disulphide Bond Formation by the Human Sulphydryl Oxidase, QSOX1.**

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Running title: *In vivo* roles and localisation of hQSOX1a.

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The abbreviations used are: ALR, augmentor of liver regeneration; AMS, 4-acetamido-4'-maleimidylstilbene-2,2'-sulphonic acid; ATCC, American type culture collection; CHO, Chinese hamster ovary; CPY, carboxypeptidase Y; DAPI, 6-diamidino-2-phenylindole; DMEM 3, Dulbecco's Modification of Eagle's medium 3; DTT, Dithiothreitol; DPS, 4,4'-dithiodipyridine; Endo H, endoglycosidase H; ER, endoplasmic reticulum; Ero1, endoplasmic reticulum oxidoreductin 1; Erv1p, endoplasmic reticulum viability 1 protein; Erv2p, endoplasmic reticulum viability 2 protein; NCO, nocodazole; NEM, N-ethylmaleimide; PDI, protein disulphide isomerase; QSOX, quiescin sulphydryl oxidase; SP, Semi-permeabilised; TGN, trans Golgi network; tPA, tissue type plasminogen activator;

## Synopsis

The discovery that the flavoprotein oxidase, Erv2p, provides oxidising potential for disulphide bond formation in yeast, has led to investigations into the roles of the mammalian homologues of this protein. Mammalian homologues of Erv2p include sulphhydryl oxidases from human lung fibroblasts, guinea-pig endometrial cells and rat seminal vesicles. Here we show that when expressed in mammalian cells the longer version of human QSOX1 protein (hQSOX1a) is a transmembrane protein localised primarily to the Golgi apparatus. We also present the first evidence showing that hQSOX1a can act as an oxidase *in vivo*. Overexpression of hQSOX1a suppresses the lethality of a complete deletion of *ERO1* in yeast and restores disulphide bond formation as assayed by the folding of the secretory protein carboxypeptidase Y.

## Introduction

Many secreted proteins contain disulphide bonds, which are required for their proper folding, function and stability. In eukaryotic cells disulphide bond formation usually occurs while the protein folds in the lumen of the endoplasmic reticulum (ER). While disulphide bonds can be formed spontaneously *in vitro* an intermediary, such as a transition metal or flavin, is required to overcome this kinetically sluggish reaction. *In vivo*, the most common mechanism for the formation of protein disulphide bonds is a thiol-disulphide exchange reaction of free thiols with an already disulphide-bonded species. These reactions are catalysed by cellular enzymes known as thiol-disulphide oxidoreductases.

The endoplasmic reticulum oxidase 1 (Ero1)-protein disulphide isomerase (PDI) dependant pathway for oxidation of disulphide bonds in substrate proteins within the eukaryotic ER is now well established [1]. PDI is the direct donor of disulphide bonds to reduced proteins and is in turn oxidised by Ero1. It is now widely accepted that Ero1 can use molecular oxygen as a terminal electron acceptor and functions with the aid of a bound FAD cofactor [2]. A second pathway for disulphide bond formation in the yeast ER was identified by screening for proteins that could rescue an Ero1p mutant when overexpressed [3]. Overexpression of a luminal ER protein, Erv2p, conferred increased resistance to DTT and the restoration of carboxypeptidase Y (CPY) maturation in both *ero1-1* and *ero1*Δ strains, leading to the realisation that Erv2p forms the basis of a disulphide bond formation pathway that can function independently of Ero1p.

Erv2p is a FAD-binding sulphhydryl oxidase and belongs to the ERV/ALR family of proteins [4]. Proteins containing an Erv2p homologous domain, known as the sulphhydryl oxidases (QSOX), have been detected in all multicellular plants and animals for which complete

genome sequences exist [5]. Two proteins belonging to the QSOX family have been discovered in humans. The first to be discovered was a gene that was up-regulated when human fibroblasts reached quiescence with the elaboration of extracellular matrix [6], hence called the Quiescin Q6 gene, now renamed as hQSOX1. Two splice variants of the human QSOX1 gene have been reported, one of 3314 bases, which encodes a peptide of 747 aa (QSOX1a), and another of 2588 bases, which encodes a peptide of 604 aa (QSOX1b). Both splice variant mRNAs are expressed at approximately the same level in human fibroblasts whilst in other cell-types the ratios vary [6]. The longer protein has a potential C-terminal transmembrane domain which has been spliced out to generate the short form of the enzyme [5]. A second gene was identified as sharing similarity with hQSOX1 and encoding another member of the QSOX family. The corresponding protein, SOXN or hQSOX2 has been studied in human neuroblastoma cells [7]. All members of the QSOX family have a thioredoxin domain fused to their C-terminal Erv2p homologous domain [5]. A second more weakly scoring thioredoxin domain lacking any redox-active disulphides adjacent to the first thioredoxin domain has been identified in human and avian QSOX sequences [8]. Thus, while the ER oxidation system requires interaction between Ero1/Erv2p and PDI, the QSOX enzymes have evolved by a fusion of these two proteins, providing them with the unique advantage of being able to introduce disulphide bonds into substrate proteins without the need to interact with additional proteins. Whilst it has been known for sometime that QSOX proteins are capable of directly introducing disulphide bonds into substrate proteins *in vitro* [9], the *in vivo* roles of these proteins still remain a mystery.

Expression and tissue distribution patterns of the rat and human QSOX enzymes indicate high levels of expression in tissues that are heavily involved in the secretion of disulphide rich proteins [5, 10, 11] leading to the hypothesis that QSOX members could be involved in the oxidative folding of secreted proteins. The intracellular location of the human and rat QSOX1 has been investigated with antibodies that recognise both the soluble and membrane associated form. The results demonstrate that whilst some intracellular material can be seen, a population of QSOX1 is secreted [6, 12, 13]. Intracellular QSOX1 proteins can be detected in the ER [5, 14], Golgi [5, 14, 15], and secretory granules [14, 15] consistent with a role for QSOX in the formation of disulphide bonds with proteins entering the secretory pathway. The widespread distribution of these enzymes in all multicellular organisms and the discovery that the yeast counterpart, Erv2p, is capable of introducing disulphide bonds into substrate proteins, independently of Ero1p, has renewed interest into the *in vivo* role of these enzymes.

In order to investigate the role of mammalian QSOX in oxidative protein folding, we created a stable cell line expressing the longer form of the human QSOX1 (hQSOX1a) enzyme as well as a model protein, human tissue type plasminogen activator (tPA). We have previously shown that overexpression of Ero1, which provides oxidising equivalents to the disulphide bond formation pathway, allows the formation of disulphide bonds in tPA to occur at concentrations of DTT which would normally result in synthesis of only the reduced form of tPA [16]. We hypothesised that if hQSOX1a was able to oxidise proteins then the overexpression of QSOX would have a similar effect on the folding of tPA. Here we describe the characterisation of the hQSOX1a protein and present the first evidence to show that this protein is capable of disulphide bond formation *in vivo*.

### Experimental Procedures

*Cell culture and treatment conditions.* Chinese Hamster Ovary (CHO) cells expressing tPA (CRL-9606, ATCC, Maryland, USA) were cultured in HAM F-12 medium (tissue culture media and supplements obtained from Invitrogen) while HT1080 (CCL-121, ATCC, USA) cells were cultured in Dulbecco's Modification of Eagle's medium 3 (DMEM 3). To disrupt the microtubule network, cells grown on coverslips were incubated at 4°C for 10 min. 5 µg/ml nocodazole (NCO, Sigma) or dimethyl sulfoxide (DMSO, solvent control, Sigma) was added in media to the cells followed by a further incubation for 2 h at 37°C.

*Construction of QSOX plasmid for mammalian expression and generation of stable cell lines.* A pINCY plasmid containing the long form of the human QSOX1 (hQSOX1a, accession number: AY358941) sequence was obtained from Genetech Inc., San Francisco, USA (a gift from Dr. Hilary F Clark, Genetech Inc., San Francisco, USA). The Invitrogen pcDNA3.1/V5-His-TOPO TA expression kit was used to construct the hQSOX1a expression plasmids. A PCR product was produced using primers that allowed amplification of all the coding sequence but removed the stop codon to allow in frame addition of the V5-His tag. The PCR product was then cloned into the pcDNA3.1/V5-His-TOPO vector. The hQSOX1a-V5-KDEL construct was created by amplification of the coding sequence minus sequence C-terminal to the beginning of the transmembrane domain. This sequence was replaced with a V5-tag in addition to a KDEL sequence at the C-terminus and cloned into pcDNA 3.1. TPA-CHO cells overexpressing QSOX and QSOX-KDEL were generated in a similar manner to tPA-CHO cells overexpressing Ero1 as described [16].

*In vitro transcription and translations-*The pcDNA3.1/V5-His-TOPO vector was linearised with *PmeI* (Roche Diagnostics) and transcribed with T7 RNA polymerase (Promega) as described [17]. hQSOX1a mRNA transcripts were translated using a rabbit reticulocyte

lysate (FlexiLysate, Promega) in the presence or absence of SP-cells as described [18]. Semi-permeabilised (SP) cells were prepared as described previously [19].

*Endoglycosidase H treatment and Proteinase K Digestion* -After translations, isolated SP cells were solubilised in 0.5% SDS, 1%  $\beta$ -mercaptoethanol for 5 min at 95°C. 1X G5 buffer (50 mM sodium citrate buffer, pH 5.5) was added and the sample was divided into two identical aliquots, which were incubated for 1 h at 37°C with either 500 units of endoglycosidase H (Endo H, NEB) or buffer alone. Cell lysates were treated with Endo H by first adding SDS and  $\beta$ -mercaptoethanol to 0.5% and 1% respectively, followed by incubation for 5 min at 95°C and treatment with enzyme as described above. Proteinase K digestions were carried out as described [18]. Samples were analyzed directly by SDS-PAGE.

*Immunofluorescence and radiolabelling*- Immunofluorescence and pulse chase analysis of tPA was carried out as described [16]. A mouse anti-V5 antibody (Upstate) was used for immunofluorescence to detect the V5-tagged hQSOX1a. Primary antibodies used for immunofluorescence included a rabbit anti-ERp57 antibody [20] as an ER marker or rabbit anti-GM130 antibody (raised against the first 73 residues at the N-terminus of GM130, a gift from Martin Lowe, The University of Manchester, UK), as a Golgi marker. Slides were viewed on an Olympus BX60 upright microscope equipped with a MicroMax cooled, slow-scan CCD camera driven by Metamorph software. For *in vivo* labelling of the V5-tagged hQSOX1a,  $2 \times 10^6$  cells per 6 cm dish were washed twice with methionine- and cysteine- free DMEM, and then pre-incubated in the same medium for 20 min at 37°C. Each monolayer was pulse-labelled with 50  $\mu$ Ci of [ $^{35}$ S]-EXPRESS label (Amersham Biosciences) for 45 minutes at 37°C and then chased with excess of methionine and cysteine for 60 min. 0.5 mM cycloheximide (Sigma) was included in the chase medium to block completion of labelled nascent chains. The cells were lysed in 1 ml of lysis buffer [50 mM Tris-HCl buffer, pH 7.4, 150 mM NaCl, 1% v/v Triton X-100, 0.1% w/v SDS, 1 mM phenylmethylsulfonyl fluoride (PMSF), 20 mM NEM] for 10 minutes on ice. Lysates were centrifuged at 12,000g for 20 min to pellet nuclei and cell debris.

*Immunoisolation of hQSOX1 from translations and cell lysates*-Each 25  $\mu$ l translation mixture was made up to 1 ml in immunoisolation buffer (50 mM Tris-HCl buffer, pH 7.4, 150 mM NaCl, 1% v/v Triton X-100). Cell lysates and translations were pre-incubated with 50  $\mu$ l protein A-Sepharose (10% w/v, Zymed laboratories Inc.) for 1 h at 4°C to pre-clear the samples of protein A binding components. Pre-cleared samples were each incubated overnight at 4°C with 1  $\mu$ l of mouse anti-V5 antibody and 50  $\mu$ l of protein A-Sepharose. The

complexes were washed three times with fresh immunoisolation buffer and then prepared for analysis by SDS-PAGE.

*Yeast strains, plasmids and growth conditions* – Yeast strains were grown at indicated temperature in YP medium (2% peptone, 1% yeast extract) containing either 2% glucose (YPD) or 2% galactose (YPGal) or in minimal medium (0.67% yeast nitrogen base, YNB) with 2% glucose or galactose, plus appropriate supplements for selective growth. Solid media were supplemented with 2% Bacto-agar. The *ERO1/ero1Δ* and *PDII/pdi1Δ* heterozygous diploid *Saccharomyces cerevisiae* strains were obtained from Steve Oliver (University of Manchester, UK). The parental wild type yeast strain (BY4742) was obtained from Barrie Wilkinson (University of Manchester, UK). The *ERO1-1* strain (CKY559) was from Chris Kaiser, (MIT, Cambridge, USA).

The coding sequence of yeast *ERV2* was amplified from yeast genomic DNA and the hQSOX1a coding sequence was amplified from the pINCY plasmid and cloned into yeast expression vector, pYES2 (Invitrogen). The *ero1Δ* and *pdi1Δ* strain containing the *P<sub>GALI</sub>-ERV2* or *P<sub>GALI</sub>-hQSOX1a* plasmid was constructed by transforming the heterozygous *ERO1/ero1Δ* and *PDII/pdi1Δ* diploid strains with *P<sub>GALI</sub>-ERV2* or *P<sub>GALI</sub>-hQSOX1a*. The transformants were sporulated and tetrads dissected on YPGal plates. As a control, the parental plasmid, pYES2, was transformed into the same strains in parallel.

*Carboxypeptidase Y Radiolabelling and Immunoprecipitations*- Yeast strains were grown in YNB medium containing 1 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and the required supplements except methionine. For pulse labelling, 25 OD<sub>600</sub> equivalents of cells were pelleted, resuspended in 2.5 ml of culture supernatant, and 500 μCi of [<sup>35</sup>S]methionine added prior to a 10 min incubation at 30°C. DTT (5 mM) was added during the labelling where indicated. Samples were chased for appropriate times by adding an excess of cold methionine and cysteine to the culture medium. For labelling in the *ERO1-1* strain, cells were grown logarithmically at 24°C and resuspended at 10 A<sub>600</sub>/ml media and preincubated at 38°C for 25 min. DTT was added to a final concentration of 62.5μM for the last 10 mins of this preincubation. Cells were labelled and chased as above but at 38°C. Labelling reactions were stopped by addition of an equal volume of ice-cold 20 mM NaN<sub>3</sub>, and incubation on ice for 5 min. CPY was immunoisolated from cell lysates using a sheep anti-CPY antibody as described [21].

*CPY redox state*-The *in vivo* redox state of CPY was assayed by modification with the thiol-reactive reagent 4-acetamido-4'-maleimidylstilbene-2, 2'-disulphonic acid (AMS, Molecular Probes). Strains were grown and radiolabelled at 30°C for 10 min as described above. Where indicated, DTT (5 mM) was added during the labelling and samples were chased for

30 min by adding an excess of cold methionine and cysteine to the culture medium. Cells were harvested by centrifugation and resuspended in 100  $\mu$ l of 20% (v/v) trichloroacetic acid. Cell membranes were disrupted by agitation with glass beads and proteins collected by centrifugation at 4°C. Protein pellets were treated with 25 mM AMS as described [22]. At the end of the AMS treatment, 1ml of immunoisolation buffer was added to each of the samples followed by immunoisolation of CPY.

*PDI redox state*-The *in vivo* redox state of PDI was assayed as described by Xiao *et al.* [23]. Yeast PDI was visualised by probing with a polyclonal anti-yeast PDI antibody (a gift from Jakob Winther, Institute of Molecular Biology and Physiology, Copenhagen, Denmark).

*Electrophoresis*-Samples for SDS-PAGE were resuspended in SDS-PAGE sample buffer (0.25mM Tris-HCl pH 6.8, 2% w/v SDS, 20% v/v glycerol, 0.004% w/v bromophenol blue). DTT (50 mM) was added to reduce samples where indicated. Proteins resolved through 10% or 7.5% gels were either transferred onto nitrocellulose for Western blotting or fixed in 10% v/v acetic acid, 10% v/v methanol, and dried. Radiolabelled products were visualized by autoradiography using Kodak Biomax MR film (GRI, Essex, UK).

## Results

*hQSOX1a-V5 is a glycosylated, transmembrane protein localised to the Golgi apparatus.*

The predicted coding sequence for hQSOX1a contains a putative N-terminal signal peptide, a conserved N-glycosylation site and a C-terminal transmembrane domain [5]. To investigate whether the protein is indeed glycosylated and transmembrane we carried out *in vitro* translation of an RNA transcript coding for V5-tagged hQSOXa in the presence of semi-permeabilised (SP) HT1080 cells [19]. When mRNA coding for hQSOX1a-V5 was translated in the absence of SP-cells, a single radiolabelled translation product of approximately 75 kDa was generated (Fig. 1A, lane 1). An additional larger product was generated when translations were carried out in the presence of cells (Fig. 1A, lane 2). This larger product was sensitive to digestion with endoglycosidase H demonstrating that hQSOX1a is glycosylated (Fig. 1B, lane 2).

To determine whether the N-terminal signal peptide of hQSOX1a results in the protein being translocated into the ER, translations were treated with proteinase K. When the hQSOX1a translation products synthesised in the presence of SP cells were treated with proteinase K (Fig. 1A, lane 3), the lower molecular weight band was digested, indicating that this was untranslocated material. However, the higher molecular weight band was partially resistant to digestion indicating that this translation product was translocated and protected from digestion. When an identical digest was performed in the presence of Triton X-100 (Fig. 1A,

lane 4) no bands were detected, demonstrating that the translation product was not intrinsically resistant to proteolysis. An increase in electrophoretic mobility of the higher molecular weight band was observed on treatment with proteinase K (Fig. 1A compare lanes 2 and 3). If the hQSOX1a gene product was anchored to the membrane by the predicted transmembrane domain then a short (21 amino acid) cytosolic domain would have been digested following proteinase K treatment potentially explaining the increased mobility of the translation product. Thus, consistent with protein prediction results for the hQSOX1a [5], translations in SP-cells showed that the hQSOX1a gene product can be glycosylated, and anchored to membranes via a C-terminal transmembrane domain.

In an attempt to determine the *in vivo* localisation and role of hQSOX1a, we generated a stable cell line expressing V5-tagged hQSOX1a and a model disulphide-bonded protein, tPA. To determine whether hQSOX1a reached the Golgi apparatus we tested the sensitivity of the intracellular protein to endoglycosidase H. Cell lysates contained a mixture of glycosylated V5-tagged hQSOX1a species some of which were sensitive to digestion with endoglycosidase H but some of which were not (Fig 1C lanes 1 and 2). The higher molecular weight species are characteristic of molecules that have had their oligosaccharides side chains modified in the Golgi apparatus. The material that remains sensitive to endoglycosidase H may also have reached the Golgi but had not been sufficiently modified to gain resistance to digestion. The result demonstrates that at steady state a proportion of the hQSOX1a synthesised is transported to the Golgi apparatus.

The endoglycosidase H experiments do not allow us to determine whether the protein is localised to post-ER compartments such as the Golgi apparatus or the cell-surface. To ascertain intracellular localisation we carried out immunofluorescence studies on the CHO-tPA cells stably expressing the hQSOX1a-V5 protein (Fig. 2A). We could not detect any hQSOX1a on the cell surface, however, a distinct perinuclear staining was observed (Fig. 2A, panel 1), which overlapped with that of the *cis*-Golgi marker protein GM130 (Fig. 2A, panel 2 and 4). Treatment of cells with nocodazole, a compound which depolymerises microtubules and hence causes disruption of the Golgi apparatus, lead to a loss of the perinuclear staining pattern and scattered cytoplasmic distribution, with both the anti-V5 and the anti-GM130 antibody (Fig. 2B), supporting the Golgi localisation of the transfected hQSOX1a.

#### *Effects of overexpression of hQSOX1a on the folding of tPA.*

While *in vitro* studies have shown that the avian QSOX and sulphhydryl oxidase from the rat seminal vesicles [24] are capable of introducing disulphide bonds into substrate proteins, the

ability of the sulphhydryl oxidases to introduce disulphide bonds into proteins within cells has not been demonstrated. Using tPA as a model protein, we wished to determine whether the expression of hQSOX1a-V5 in the CHO-tPA cells would have any effect on the folding of tPA. We have previously shown that conditions preventing disulphide bond formation, such as addition of the reducing agent DTT to the culture medium of living CHO-tPA cells, leads to the complete glycosylation of a sequon within tPA that would otherwise undergo variable glycosylation [25]. However the overexpression of Ero1 conferred an increased resistance to the effects of reduction with DTT on tPA glycosylation and disulphide bond formation [16]. To determine whether the expression of hQSOX1a could function like Ero1 *in vivo*, hQSOX1a-V5 transfected stable CHO-tPA cells were radiolabelled for 10 min in the absence or presence of varying concentrations of DTT. At the end of the labelling period, the cells were treated with the membrane permeable alkylating agent NEM to block free thiols, lysed and tPA immunisolated. The immunisolated material was then subjected to both reducing and non-reducing SDS-PAGE (Fig. 3).

In the untransfected CHO-tPA cells, the tPA synthesized during the 10 min labelling period in the absence of DTT migrated as a doublet band when reduced prior to electrophoresis (Fig. 3A, upper panel, lane 1) indicating that variable glycosylation of tPA had occurred. When subjected to non-reducing SDS-PAGE (Fig. 3A, lower panel, lane 1) tPA migrated with a greater mobility and with a more diffuse banding pattern than on reducing gels, indicating that in the absence of DTT, tPA forms intrachain disulphide bonds. As the concentration of DTT in the labelling medium was increased from 0 to 5 mM, the doublet pattern progressively disappeared until only a single band of the over glycosylated (type 1) tPA was present at concentrations of DTT above 0.75 mM (Fig. 3A, upper panel, lanes 2-10). This change in banding pattern was accompanied by a progressive reduction in the formation of disulphide bonds as indicated by the slower migrating sharper bands under non-reducing conditions (Fig. 3A, lower panel, lanes 2-10). Thus, at 1 mM DTT no disulphide bonds were formed as no faster migrating bands were seen when the sample was run under non-reducing conditions (Fig. 3A, lane 8). A clear difference in mobility of type 1 tPA synthesised in the absence or presence of 5 mM DTT (Fig. 3A, upper panel, lanes 1 and 10) and separated under reducing conditions was observed. This is because the tPA synthesised in the presence of 5 mM DTT is completely reduced, thus making its 34 cysteine residues available for alkylation by NEM, slowing the migration of the protein. The lower intensity of signal at higher concentrations of DTT is due to the antibody having a lower affinity during immunisolation for the reduced and alkylated protein than the disulphide bonded form as characterised previously [16].

Similar results were obtained in the CHO-tPA cells expressing the V5-tagged hQSOX1a (Fig. 3B). This is in contrast to results obtained with the overexpression of Ero1 [16]. In the cell line overexpressing Ero1-L $\alpha$  variable glycosylated tPA could be observed at DTT concentrations as high as 1mM and formation of disulphide bonds even at 2 mM. Thus, unlike Ero1 overexpression, the overexpression of QSOX1a had little or no effect on the glycosylation or disulphide bond formation of tPA.

One explanation for the lack of effect of hQSOX1a overexpression on the disulphide bond formation within tPA may be that most of the exogenously expressed hQSOX1a-V5 was localised in the Golgi, while the folding and glycosylation of tPA occurs in the ER. To check whether the QSOX1a overexpression would provide resistance to DTT if the hQSOX1a-V5 was located in the ER, we created a CHO-tPA cell-line stably expressing hQSOX1-V5 with a C-terminal KDEL sequence for ER localisation. Immunofluorescence studies on CHO-tPA cells stably expressing hQSOX1-V5-KDEL showed that the V5- tagged hQSOX1 lost its perinuclear staining and instead showed a characteristic ER like distribution (Fig. 4A). A similar staining pattern was seen when cells were stained with the ER marker ERp57 (Fig. 4A). Thus replacement of the transmembrane and cytosolic tail sequence with a -KDEL sequence caused redistribution of the hQSOX1-V5 from the Golgi into the ER, in turn trapping the newly synthesised tPA and the exogenously expressed hQSOX1a in the same cellular compartment.

In order to determine whether the trapping of the hQSOX1a-V5 in the ER has any effect on disulphide bond formation within tPA, cells stably expressing hQSOX1-V5-KDEL were assayed for tPA folding as described above. In the hQSOX1a-V5-KDEL expressing cells disulphide bond formation is as sensitive to the effects of DTT as in cells without hQSOX1a (compare Fig. 4B with Fig. 3A). Previous experiments looking at the *in vitro* activity of Ero1 and QSOX have shown that both enzymes are fully active at the DTT concentrations used in our experiments [26, 27]. Based on our results we can conclude that, unlike Ero1, the hQSOX1a expressed in the CHO-tPA does not contribute to the formation of disulphide bonds during folding of tPA. Given the localisation of hQSOX1a it is possible that the mammalian QSOX enzymes are not required for the initial steps of disulphide bond formation that occur in the ER, but rather in the formation of disulphide bonds within the Golgi apparatus.

#### *Overexpression of hQSOX1a can rescue an ero1 $\Delta$ strain*

The inability to detect a role for QSOX in facilitating disulphide bond formation within mammalian cells led us to hypothesise that similar to observations made in yeast, oxidation

of proteins via Ero1 could be the major pathway for disulphide formation in the mammalian ER [28]. Thus the contribution of QSOX to the disulphide bond formation pathway may only be evident in cells with an impaired Ero1 function. To explore this possibility a heterozygous diploid *ERO1/ero1Δ* strain was transformed with a yeast expression plasmid containing the hQSOX1a coding sequence under the control of the GAL1 promoter. The strain was also transformed with an empty plasmid to serve as a negative control and plasmid containing the *ERV2p* coding sequence to serve as a positive control. The transformants were selected, allowed to sporulate and tetrads were dissected onto glucose and galactose media. At least 60 tetrads were dissected for each strain with identical results obtained for each tetrad. As reported previously [3], all the spores derived from the *ERV2* transformants were able to grow on galactose media, while only 2 of the 4 spores from the strain transformed with the empty plasmid were alive (results not shown). When transformants containing the *P<sub>GALI</sub>-hQSOX1a* plasmids were sporulated, all four spores were found to be viable on galactose media (results not shown), although the *ero1Δ P<sub>GALI</sub>-hQSOX1a* strain grew more slowly than the *ero1Δ P<sub>GALI</sub>-ERV2* strain (Fig. 5A, left panel). Growth of the *ero1Δ P<sub>GALI</sub>-hQSOX1a* strain depended on the overexpression of *hQSOX1a* as this strain could not grow on glucose medium (Fig. 5A, right panel). These results demonstrate that hQSOX1a is capable of rescuing Ero1 deficient strains and is therefore capable of fulfilling the essential function of Ero1 within yeast cells.

To determine whether the overexpression of hQSOX1a is able to restore disulphide bond formation in the *ero1Δ P<sub>GALI</sub>-hQSOX1a* strain we evaluated the folding and transport of carboxypeptidase Y (CPY). CPY contains five intrachain disulphide bonds whose formation is necessary for its folding and exit from the ER [29]. Three sub-cellular forms of CPY can be detected by reducing SDS-PAGE during its folding and maturation within the cell [30]: a 67 kDa ER form termed p1CPY, a 69 kDa Golgi form termed p2CPY and finally a 61 kDa mature (m) vacuolar form of the protein. When disulphide bond formation is compromised, only the p1 form of CPY is synthesised due to the absolute requirement for disulphide bonds to form prior to transport of CPY from the ER to the Golgi apparatus [29]. In the wild type cells most of the CPY synthesised in the ER in the absence of DTT is efficiently converted to the p2 form and finally to the mature form within 20 min of the chase time (Fig. 5B, lane 3), demonstrating correct folding, disulphide bond formation and transport of CPY in this strain. The mature form of CPY can also be detected in the *ero1Δ P<sub>GALI</sub>-ERV2* and the *ero1Δ P<sub>GALI</sub>-hQSOX1a* strains, although it takes about 45 min for most of the CPY in these strains to be processed, possibly due to the slow growing nature of these strains (Fig. 5B, lanes 2-5). CPY processing was also assessed in a temperature sensitive strain of Ero1 grown at the non-

permissive temperature (Fig. 5C, lanes 2-5). As shown previously [31], no processing was seen in the *Ero1-1* strain grown at the non-permissive temperature demonstrating that the lack of a functional *Ero1* results in a lack of processing of CPY to the mature protein. In contrast folding of CPY in the *ero1Δ P<sub>GALI</sub>-hQSOX1a* strain occurred at 38°C with faster kinetics than at 30°C (compare Fig. 5B and 5C). These results demonstrate that like *Erv2p*, *hQSOX1a* is capable of reversing the *Ero1* defect and therefore supports disulphide bond formation in an *Ero1p* independent pathway. Addition of 5 mM DTT to the media during the pulse period results in accumulation of the p1 form of CPY in all three strains (Fig. 5B, right panel, lane1). As observed previously [32], on removal of the DTT in the wild type yeast strains, the majority of the CPY is able to fold and reach its mature form within 45 min (Fig. 5B, top right panel, lanes 2-5). In contrast, the CPY in the *ero1Δ P<sub>GALI</sub>-ERV2* and the *ero1Δ P<sub>GALI</sub>-hQSOX1a* strains were unable to resume folding on removal of the DTT and accumulated in the immature ER form (Fig. 5B, middle right and lower right panels).

The accumulation of CPY in the ER form could either be due to the inability of the strains to recover from DTT treatment or due to the formation of non-native disulphide bonds in CPY on removal of the DTT. To address this point the oxidation state of CPY was assayed directly by modification of free thiols with the thiol conjugating reagent AMS. Modification of a single cysteine residue by AMS increases the molecular mass of the protein by approximately 0.5 kDa [33]. When the wild type yeast was radiolabelled for 10 min in the absence of DTT and chased for 30 min only a slight shift in mobility was observed following AMS treatment (Fig. 6A, lanes 1,2). This is consistent with modification of the single cysteine present in oxidised CPY [34] indicating that CPY was fully oxidised under these conditions. Similar results were obtained with the *ero1Δ P<sub>GALI</sub>-ERV2* and *ero1Δ P<sub>GALI</sub>-hQSOX1a* strains (results not shown). In contrast, when 5 mM DTT was added to the pulse medium and the cells were allowed to recover from DTT for 30 min, only the CPY in wild type cells was able to form disulphide bonds (Fig. 6A, lane 5). The CPY from the *ero1Δ P<sub>GALI</sub>-ERV2* and *ero1Δ P<sub>GALI</sub>-hQSOX1a* strains was modified with AMS to the same extent as the fully reduced CPY (Fig. 6A, compare lanes 6 and 7 with lane 4) indicating that the removal of DTT in these strains does not result in rapid re-establishment of oxidising conditions within the ER. Thus, although *Erv2p* and *hQSOX1a* overexpression can rescue *Ero1p* deficient strains, challenging the ER in such strains with reducing agents seems to abolish their ability to carry out disulphide bond formation in substrate proteins, even after removal of the reducing agent.

#### *Interaction of hQSOX1a with Pdi1p*

The presence of a PDI-like thioredoxin domain in addition to an FAD binding domain within the QSOX sequence has led to the suggestions that unlike Ero1, the QSOX family of proteins have evolved to be able to interact directly with substrate proteins [5]. Indeed, it has been shown that *in vitro* QSOX1 is able to directly oxidise proteins in the absence of PDI [24]. To determine whether hQSOX1a can function *in vivo* in the absence of PDI, *PDI1/pdi1Δ* heterozygous diploid yeast strains were transformed with *P<sub>GALI</sub>-ERV2* and *P<sub>GALI</sub>-hQSOX1a* plasmids. Transformants were selected, sporulated and tetrads dissected onto glucose and galactose media. Only spores containing the chromosomal *PDI1* were able to grow (results not shown), indicating that despite the ability of Erv2p and QSOX1 to carry out direct oxidation of substrate proteins, there is a requirement for PDI to be present for these cells to be viable. This result supports *in vitro* data obtained for the avian QSOX1a, where increased yields of RNase A were obtained by incorporation of reduced PDI into the reaction [24].

Both QSOX1 and Erv2p have been shown to oxidise PDI *in vitro* [3, 24] and Ero1p has been shown to be necessary to maintain PDI in an oxidised state *in vivo*. To evaluate the possibility that hQSOX1a was acting through Pdi1p in the *ero1Δ P<sub>GALI</sub>-hQSOX1a* strain we determined the redox state of Pdi1p within these cells. Mal-PEG was used to determine the *in vivo* redox state of yeast PDI as described by Xiao *et al* [23]. Yeast PDI has six cysteine residues, four in the active sites and two which form a stable intrachain disulphide bond [23, 35]. The predominant bands in the wild-type strain represent fully oxidised or reduced Pdi1p and a species shifted by two Mal-PEG modifications (Fig. 6C, lane 3) representing partially reduced Pdi1p [23]. Pdi1p from the *ero1Δ P<sub>GALI</sub>-hQSOX1a* and *ero1Δ P<sub>GALI</sub>-ERV2* was much more reduced as bands corresponding to 2, 4 and 6 cysteine modifications by Mal-PEG can be detected in these strains (Fig. 6C, lanes 4 and 5). Hence both the active site and the structural disulphide are more reduced in the complemented strains compared to the wild-type. The presence of increased amounts of reduced Pdi1p in these strains would indicate that both Erv2p and hQSOX1a are not as efficient as Ero1p in oxidising Pdi1p *in vivo*. The lack of formation of the structural disulphide within a proportion of the Pdi1p present in these strains would affect overall activity as this disulphide has been shown to destabilise the N-terminal active site [35]. Such destabilisation improves both the oxidase and isomerase activities of Pdi1p and could provide a possible explanation for the observed slow growth of these two strains.

## Discussion

Previous studies on the QSOX family of enzymes have demonstrated their ability to introduce disulphide bond into proteins *in vitro* [24]. However, a demonstration of enzymatic

activity *in vivo* has been missing mainly due to a lack of knowledge of potential endogenous substrates. Here we show that hQSOX1a is able to complement the function of Ero1 at least in yeast. The ability to act as a replacement for Ero1 under these circumstances does not necessarily mean that the enzyme acts as an oxidase within the ER of mammalian cells. Indeed the lack of effect of overexpression of the protein on the sensitivity of the oxidative folding pathway of tPA to a reducing agent coupled with a cellular localisation to the Golgi apparatus would argue against such a function within the ER.

Most disulphide bonded proteins are normally folded and assembled in the endoplasmic reticulum (ER) and transported to other compartments of the secretory pathway in a fully disulphide bonded form. The remarkable distribution of proteins of the QSOX family in many cellular compartments [5] indicates that new pathways for disulphide bond formation outside the eukaryotic ER remain to be investigated. The approach we have taken to study the localisation of the hQSOXa form differs from previous approaches in that we specifically detect the transmembrane version rather than both the transmembrane and soluble proteins. Hence we see a clear localisation in the Golgi apparatus suggesting that substrate proteins may exit the ER in a precursor form that requires further disulphide bond formation prior to, or during secretion. Identification of the substrates for QSOX enzymes, will be crucial if we are to fully understand the role of these enzymes within the cell. Candidates proteins would be components of the extra-cellular matrix which are typically high molecular weight containing several disulphide bonds which mature at later stages of the secretory pathway often forming higher order structures stabilised by disulphide bonds. One characteristic of the disulphides that would be formed by QSOX in these proteins is that they would be either between separate polypeptide chains or between already folded proteins domains. Hence the chances of formation of the incorrect disulphide bonds would be minimal and therefore so would be the need for an isomerase.

While hQSOX1a may be involved in the formation of disulphide bonds within the cell or on the cell surface, the shorter QSOX1 form (hQSOX1b) may function as a secreted oxidase to counteract the effects of extracellular reducing agents. The overall homology between the QSOX2 and the QSOX1 proteins is ~40% with 68% identity in functional regions such as the thioredoxin-like or the ERV-1 domain [7]. While initial studies indicate that hQSOX2 plays an important role in inducing apoptosis in neuroblastoma cells, it will be interesting to determine whether it can also act as an oxidase. While hQSOX1a is highly expressed in the placenta, stomach and lung [5], high levels of hQSOX2 were detected in the pancreas, brain, kidney and heart [7]. Thus it is possible that the two genes have different functions or take on redundant functions in different tissues.

Unlike Ero1 which acts through PDI [22], QSOX does not require any additional partners to introduce disulphides into proteins. However, the ability of QSOX to oxidise substrate proteins, rapidly and indiscriminately as demonstrated *in vitro* [24] would potentially make them unsuitable proteins to have in the ER. In addition to newly synthesised reduced proteins which have to be oxidised to their native forms, the ER is also the site at which misfolded proteins are reduced and targeted for degradation [36]. QSOX could potentially catalyse the oxidation of both these kinds of substrates. The presence of a strong, indiscriminate oxidant will thus act as a load rather than a help in the ER as the cell would need to continuously isomerise the non-native disulphides introduced into substrate proteins. As the product of the reaction of QSOX with substrate proteins is hydrogen peroxide [24], such futile formation and isomerisation of disulphide bonds could lead to the oxidative stress, ultimately leading to cell-death. However, our results with tPA would suggest that QSOX has a minimal role in catalysis of disulphide bonds within the ER. The presence of a robust Ero1-dependent pathway for disulphide bond formation could prevent QSOX from playing a major role in disulphide bond formation within the ER under normal physiological conditions. Thus, while Ero1 and PDI are involved in oxidative folding of secretory proteins in the ER of eukaryotic cells, it is likely that QSOX acts at later stages of the secretory pathway, either to maintain disulphide bonds or to oxidise specific substrates.

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### Figure legends.

**Figure 1: *In vitro* translations and *in vivo* processing of hQSOX1a.** (A): hQSOX1a-V5 mRNA was translated in a rabbit reticulocyte lysate in the absence (lane 1) or presence of SP HT1080 cells (lane 2-5) for 1 h at 30°C. Isolated cells were treated with proteinase K (lane 3) with the addition of 1% (w/v) Triton X-100 (TX 100) as indicated (lane 4). (B): hQSOX1a-V5 mRNA was translated in a rabbit reticulocyte lysate in the presence of SP HT1080 cells for 1 h at 30°C. Isolated cells were incubated with (lane 2) or without (lane 1) Endo H at 37°C for 1 h. (C): Cells stably expressing hQSOX1a-V5 were lysed and proteins were either treated (lane 1) or not treated (lane 2) with endoglycosidase H. Proteins were detected by immunoblotting with V5-antibody.

**Figure 2: Intracellular localisation of V5-tagged hQSOX1a.** (A): hQSOX1a-V5 expressing CHO-tPA cells were fixed and simultaneously stained with mouse anti-V5 antibody and rabbit anti-GM130 antibody. Secondary antibodies were conjugated to Alexa fluor 448 (green) or Alexa fluor 594 (red). Cell nucleus was stained with DAPI. (B): Golgi localisation of the hQSOX1a-V5 was confirmed by incubating cells in the presence of 5 µg/ml nocadazole for 2 h. Cells were then fixed and stained as above.

**Figure 3: Effect of hQSOX1a expression on the folding and glycosylation of tPA in the presence of DTT.** (A) Untransfected or (B) stable cell line expressing hQSOX1a-V5 was radiolabelled in the absence (lane 1) or presence (lanes 2-10) of varying concentrations of DTT. Cells were lysed and newly synthesised tPA immunisolated with anti-tPA antibody. Immunisolated proteins were separated by SDS-PAGE under reducing (upper panel) and non-reducing (lower panel) conditions.

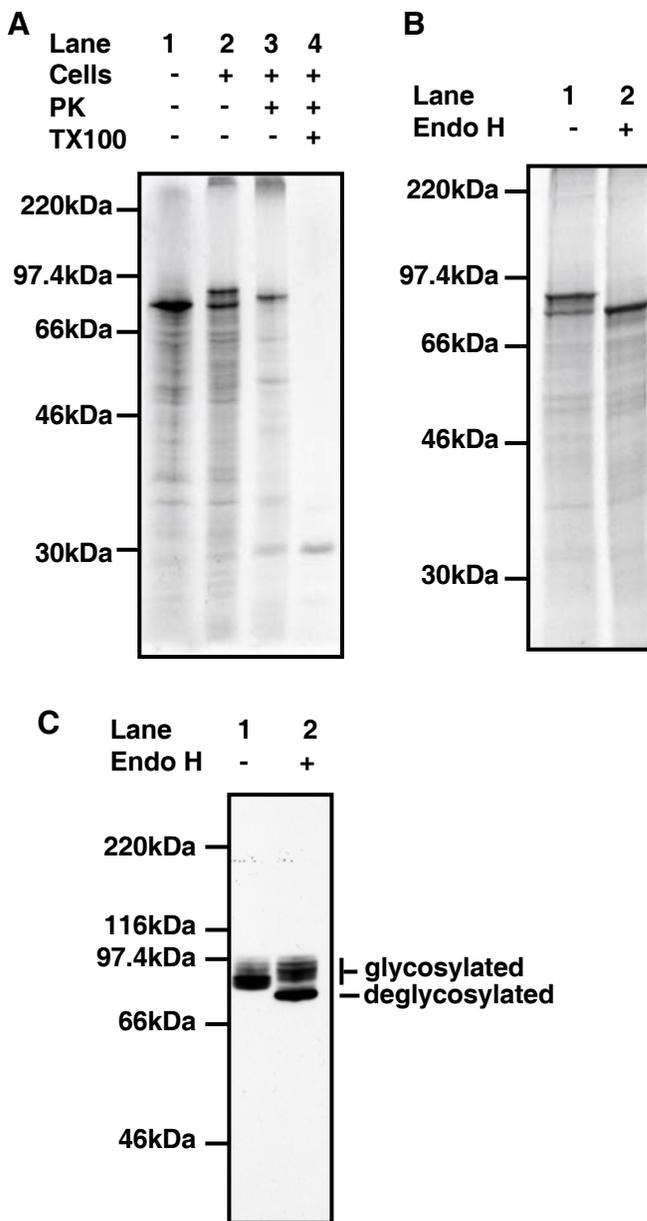
**Figure 4: Localisation of hQSOX1a-V5-KDEL in CHO-tPA cells to the ER.** (A): CHO-tPA cells stably expressing hQSOX1-V5-KDEL were fixed and stained with a mixture of anti-V5 antibody (left panel) and anti- ERp57 antibody (right panel). Secondary antibodies were conjugated to Alexa fluor 448 (green) or Alexa fluor 594 (red). Cell nuclei were stained with DAPI. (B) CHO-tPA cells expressing hQSOX-V5-KDEL were radiolabelled for 10 min in the absence (lane 1) or presence (lanes 2-10) of varying concentrations of DTT. Cells were lysed and tPA immunisolated from lysates with anti-tPA antibody. Immunisolated proteins were separated by SDS-PAGE under reducing (upper panel) and non-reducing conditions (lower panel).

**Figure 5: hQSOX1a overexpression restores growth and formation of disulphide bonds in an Ero1p deficient strain.** (A): Growth of *wt* or an *ero1Δ* strain containing  $P_{GALI}$ -*hQSOX1a* or  $P_{GALI}$ -*ERV2I I* on YPGal (left panel). Growth is dependant on overexpression of Erv2p or hQSOX1a as the strains could not grow on glucose medium (right panel). (B): The parental BY4742 (*wt*), *ero1Δ P<sub>GALI</sub>-hQSOX1a* and *ero1Δ P<sub>GALI</sub>-ERV2I I* strains were radiolabelled in the absence (left panels) or presence of 5 mM DTT (right panels). Labelling was stopped, cells allowed to recover in the absence of DTT and samples taken at various time points as indicated. Cells were lysed and CPY folding analysed by separating the immunisolated proteins by SDS-PAGE under reducing conditions. Positions of the ER (p1), Golgi (p2) and vacuolar (m) forms of CPY are indicated. (C) The *ERO1-I* (CKY559) or the *ero1Δ P<sub>GALI</sub>-hQSOX1a* strain growing logarithmically at 24°C were resuspended at 10 A<sub>600</sub>/ml and incubated at 38°C. After 15 min, DTT was added to 65µM and after a further 10

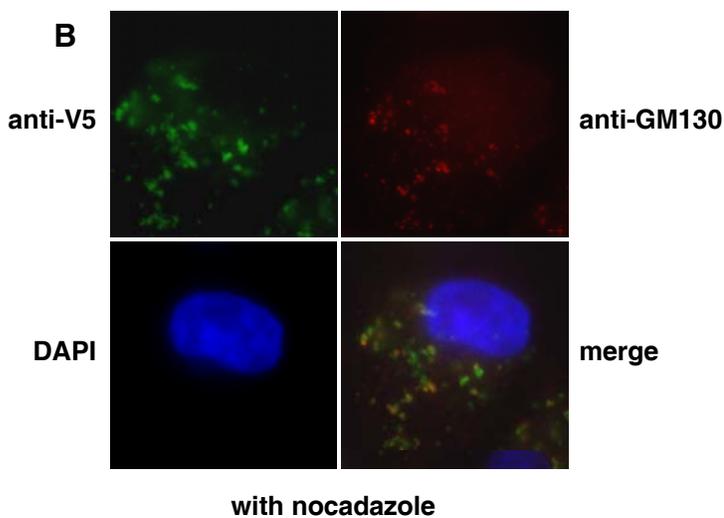
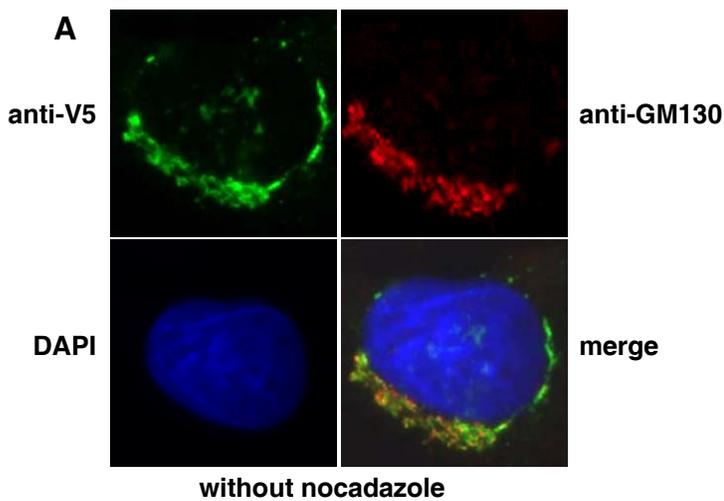
min cells were radiolabelled and then chased for the indicated times. Cells were lysed and CPY folding analysed as in (B).

**Figure 6: Redox states of CPY and PDI.** (A): BY4742 *wt* cells were radiolabelled for 10 min in the absence (lanes 1 and 2) or presence of 10 mM DTT (lanes 3 and 4) to detect the fully oxidised and fully reduced p1CPY respectively. BY4742 (*wt*), *ero1Δ P<sub>GALI</sub>-hQSOX1a* (QSOX) and *ero1Δ P<sub>GALI</sub>-ERV2* (Erv) were radiolabelled for 10 min in the presence of 5mM DTT followed by a 30 min chase in the absence of DTT (lanes 5, 6 and 7). Cells were isolated at the end of the labelling and chase periods and proteins from cell pellets were TCA precipitated and solubilised in the presence (lanes 2, 4-7) or absence (lanes 1, 3) of 25 mM AMS prior to immunoisolation of CPY. Samples were resolved by non-reducing SDS-PAGE. The reduced (red) and oxidised (ox) forms of p1CPY are indicated. (B): Trichloroacetic acid (20%) was added to logarithmically growing cultures to quench thiol/disulphide exchange and precipitate proteins. Free sulphhydryl groups were modified by resuspension in 5 mM Mal-PEG under denaturing conditions (3% SDS, 0.2 M Tris-HCl, pH 8). Proteins were separated by SDS-PAGE and visualized by Western blotting using an anti-yeast PDI antibody. Apparent molecular weight shifts above the unmodified protein indicate the number of free sulphhydryl groups modified. The redox states of PDI in the parental BY4742 (*wt*), *ero1Δ P<sub>GALI</sub>-hQSOX1a* (QSOX) and *ero1Δ P<sub>GALI</sub>-ERV2* (Erv) are compared with reduced (*red*) and oxidized (*ox*) controls that were treated with 10 mM DTT or 1 mM diamide before Mal-PEG modification.

**Figure 1**

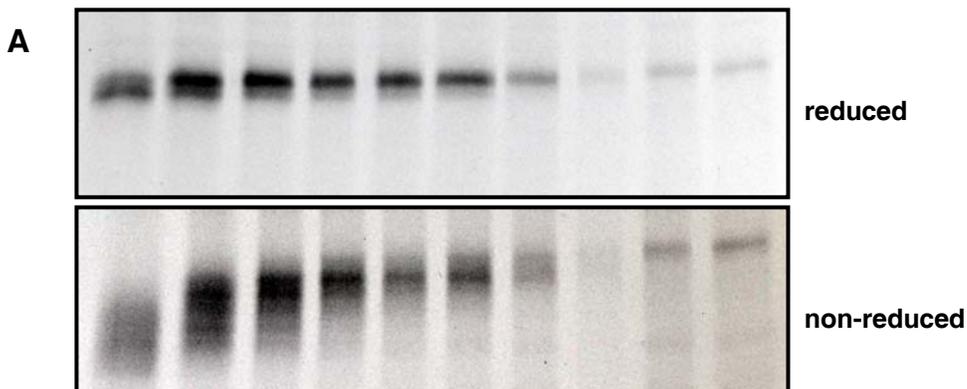


**Figure 2**

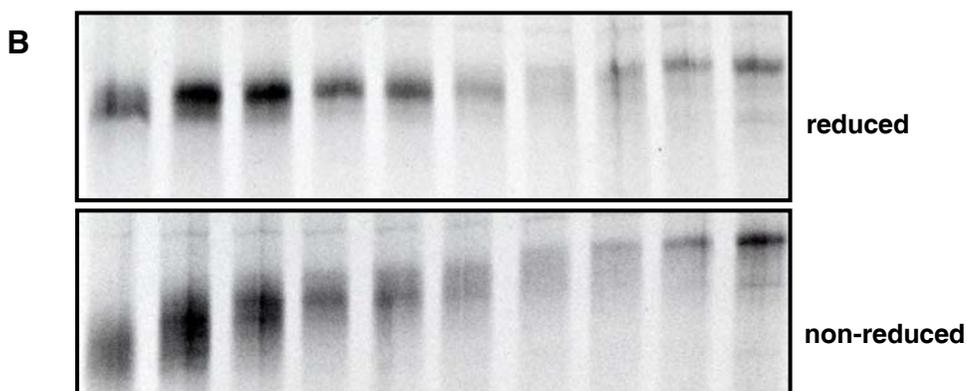


**Figure 3**

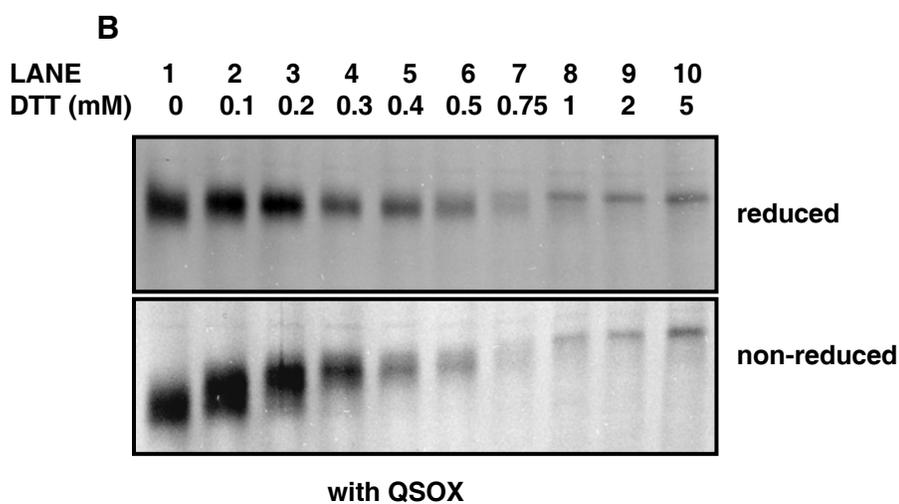
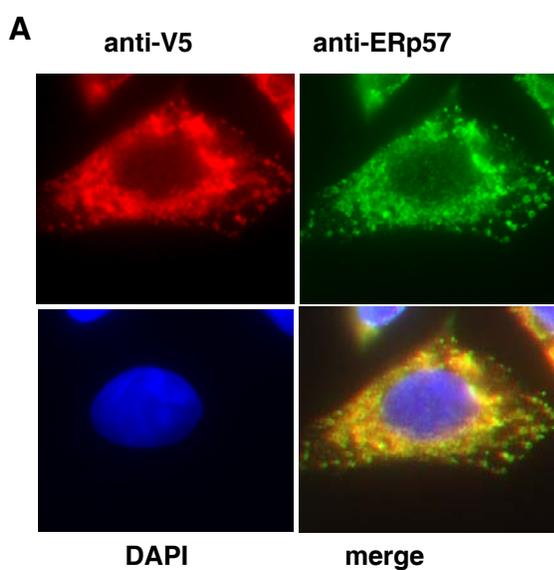
<b>LANE</b>	1	2	3	4	5	6	7	8	9	10
<b>DTT (mM)</b>	0	0.1	0.2	0.3	0.4	0.5	0.75	1	2	5



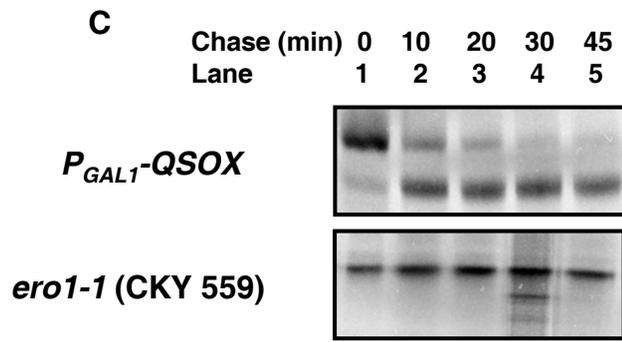
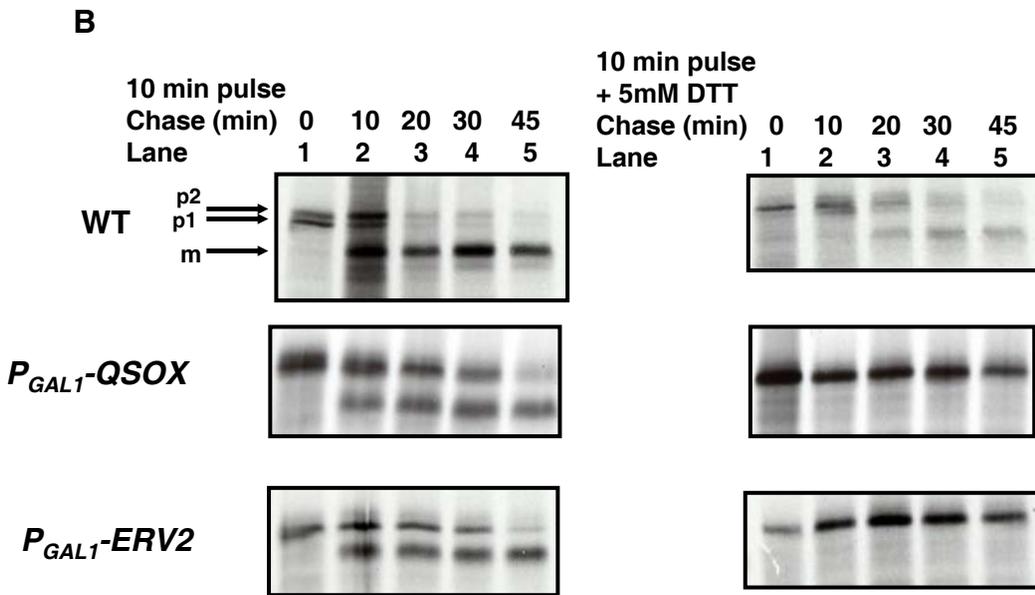
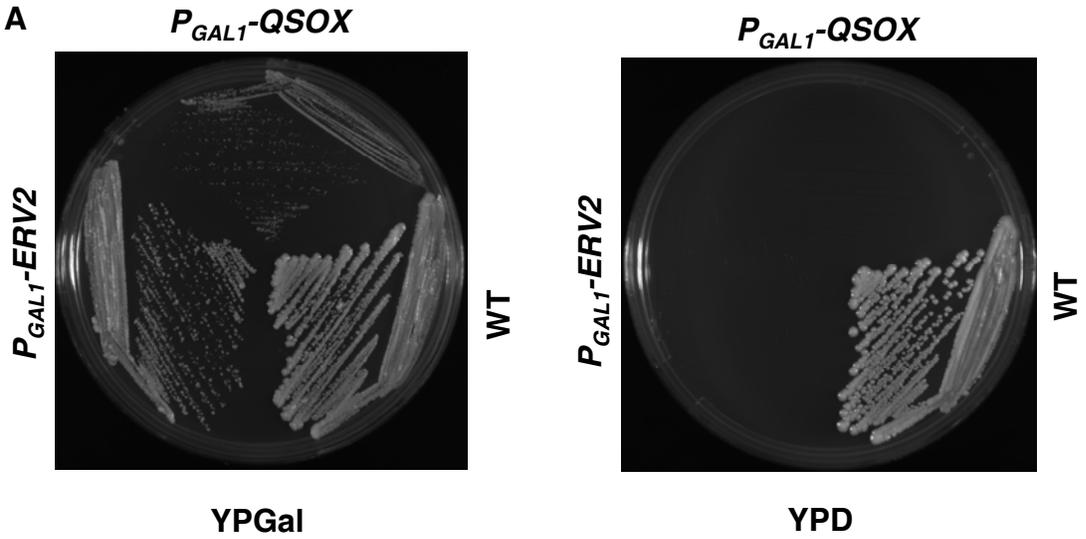
<b>LANE</b>	1	2	3	4	5	6	7	8	9	10
<b>DTT (mM)</b>	0	0.1	0.2	0.3	0.4	0.5	0.75	1	2	5



**Figure 4**

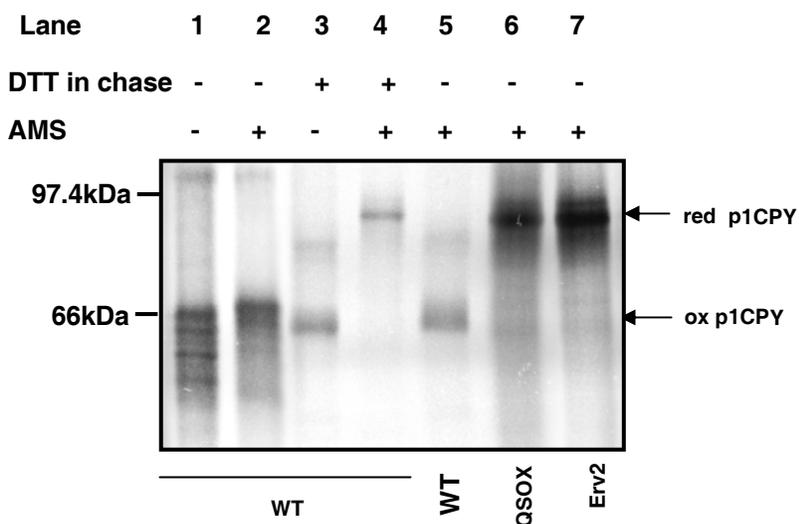


**Figure 5**



**Figure 6**

**A**



**B**

