A novel disulphide switch mechanism in Ero1 α balances ER oxidation in human cells

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Oxidative maturation of secretory and membrane proteins in the endoplasmic reticulum (ER) is powered by Ero1 oxidases. To prevent cellular hyperoxidation, Ero1 activity can be regulated by intramolecular disulphide switches. Here, we determine the redox-driven shutdown mechanism of Ero1 α , the housekeeping Ero1 enzyme in human cells. We show that functional silencing of Ero1 α in cells arises from the formation of a disulphide bond—identified by mass spectrometry—between the active-site Cys⁹⁴ (connected to Cys⁹⁹ in the active enzyme) and Cys¹³¹. Competition between substrate thiols and Cys¹³¹ creates a feedback loop where activation of Ero1 α is linked to the availability of its substrate, reduced protein disulphide isomerase (PDI). Overexpression of Ero1 α -Cys131Ala or the isoform Ero1 β , which does not have an equivalent disulphide switch, leads to augmented ER oxidation. These data reveal a novel regulatory feedback system where PDI emerges as a central regulator of ER redox homeostasis.

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Introduction

The endoplasmic reticulum (ER) maintains redox conditions that are optimal for the essential process of native disulphide-bond formation in proteins of the secretory pathway. ER redox homeostasis is achieved by an interplay between the ER-localized disulphide-generating oxidases of the Ero1 family (Sevier and Kaiser, 2008) and reduced glutathione (GSH) that transfers reducing equivalents from the cytosol into the ER (Cuozzo and Kaiser, 1999; Molteni *et al*, 2004). By competing with nascent polypeptides for Ero1-derived oxidizing equivalents, GSH is oxidized to glutathione disulphide (GSSG) in the ER lumen. Together, GSH and GSSG form a

redox buffer that helps to maintain the ER redox homeostasis. Thus, redox-dependent processes such as oxidative protein folding are hampered under conditions of glutathione depletion (Cuozzo and Kaiser, 1999; Chakravarthi and Bulleid, 2004; Molteni *et al*, 2004).

In principle, Ero1-catalysed oxidation can lead to undesirable, constant consumption of cellular GSH (Thorpe and Coppock, 2007). Moreover, potentially cytotoxic reactive oxygen species (ROS) accumulate as a side product of Ero1 activity that generates stoichiometric amounts of H₂O₂ per newly formed disulphide (Gross *et al*, 2006). ER oxidation therefore requires control mechanisms that link the process of disulphide-bond generation to the overall redox conditions in the ER. The recent detection of two regulatory disulphide bridges in *Saccharomyces cerevisiae* Ero1 (Ero1p) that must be reduced for catalytic activity has provided the first direct evidence for such a feedback system (Sevier *et al*, 2007). The question of the physiological reductant(s) of the Ero1p disulphide switches, that is, the redox sensing mechanism of the enzyme, has not yet been resolved.

The oxidases of the Ero1 family are flavoproteins. They harbour two di-cysteine active-site motifs that cooperate in the transfer of electrons from substrate to the flavin adenine dinucleotide (FAD) cofactor: an outer active site (also termed the 'shuttle cysteines') interacting with substrate, and an inner active site that is FAD adjacent (Sevier and Kaiser, 2008). The shuttle cysteines are located in a flexible polypeptide segment that can reach into the helical core domain and shuttle electrons to the inner active site (Gross *et al*, 2004). In Ero1p, the regulatory disulphides (Cys⁹⁰–Cys³⁴⁹ and Cys¹⁵⁰–Cys²⁹⁵) link the flexible loop region to the core domain.

In human cells, two Ero1 isoforms exist that are both transcriptionally upregulated under conditions of ER stress (Pagani *et al*, 2000; Marciniak *et al*, 2004). Whereas the Ero1 α protein is expressed in most cell types, detectable protein levels of Ero1 β are found only in select tissues (Dias-Gunasekara *et al*, 2005). Ero1 α exists as two oxidized redox forms *in vivo*, OX1 and OX2, that differ in their electrophoretic mobility on non-reducing gels (Benham *et al*, 2000). As the faster-migrating OX2 form is favoured under oxidizing conditions and disfavoured upon treatment with a reducing agent (Anelli *et al*, 2002; Molteni *et al*, 2004), it appears to comprise at least one additional long-range disulphide bond as compared with the slower-migrating OX1 species. For an overview of the cysteines in Ero1 α and Ero1p, see Figure 2B.

Apart from the monomeric OX1 and OX2 forms of Ero1 α , a band of ~130 kDa representing a covalent complex between Ero1 α and protein disulphide isomerase (PDI) linked by an inter-chain disulphide has been identified by non-reducing SDS-PAGE (Benham *et al*, 2000; Mezghrani *et al*, 2001). PDI is the only conserved substrate in mammalian cells and yeast (Frandsen and Kaiser, 2000; Tu *et al*, 2000) and is generally believed to be the primary acceptor of disulphide bonds from Ero1. Thus, one function of PDI is to oxidize nascent polypeptides (Appenzeller-Herzog and Ellgaard, 2008b). Similar

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to its close homologue ERp57, PDI has two redox-active domains—**a** and **a'**—that each feature a Cys-Gly-His-Cys active-site sequence for the catalysis of thiol–disulphide exchange. In HEK293 cells, PDI is predominantly reduced ($\sim 50\%$ of all molecules) at steady state, but fully oxidized ($\sim 16\%$) and semi-oxidized (oxidized in one active site only, $\sim 33\%$) species are also present (Appenzeller-Herzog and Ellgaard, 2008a).

Here, we describe the molecular basis of the OX1–OX2 redox switch in Ero1 α that directly regulates enzyme activity *in vivo*. Furthermore, we show that the activation state of Ero1 α is coupled to the availability of reduced PDI, demonstrating the existence of a regulatory feedback loop in ER redox control.

Results

Overexpressed Ero1 variants are active, but elicit only minimal ER stress

Our studies of ER redox regulation by Ero1 necessitated a well-controlled system for ectopic inducible expression of human Ero1 α and Ero1 β . For this purpose, we used a recombinase-mediated integration system to generate four HEK293-based cell lines. In addition to cells encoding myc- and His-tagged wild-type Ero1 α and Ero1 β , we created cells expressing the two Ero1 α mutants Cys131Ala (C131A) and Cys394Ala (C394A). As described in further detail below, the former cannot build a critical regulatory disulphide, whereas the latter carries a mutation in the inner active site and has been suggested to be a dominant-negative mutant (Mezghrani *et al*, 2001). Western blot analysis of cells untreated or induced with doxycyclin for 24 and 48 h confirmed doxycyclin-dependent expression of all constructs (Figure 1A). As expected for stable cell clones generated using a system for gene integration at a single genomic location (O'Gorman *et al*, 1991), the recombinant Ero1 proteins were expressed at similar levels. Compared with the levels of endogenous Ero1 α , induction of Ero1 α cells for 24 or 48 h led to approximately 20-fold or 30-fold overexpression, respectively (Supplementary Figure S1).

Overexpression of Ero1 holds the potential to increase the cellular load of ROS (Harding *et al*, 2003; Haynes *et al*, 2004). In higher eukaryotes, such ER-derived oxidative stress is counteracted by activation of the ER kinase PERK that triggers two signalling pathways—Nrf2 (Cullinan and Diehl, 2004) and eIF2 α (Harding *et al*, 2003)—both leading to an increase in the cellular antioxidant capacity through increased glutathione synthesis. We therefore asked if overexpression of Ero1 in our cell lines activated PERK signalling. Lysates of induced Ero1 cells were analysed by western blotting using antibodies specific for activated (phosphorylated) PERK and eIF2 α . In none of the Ero1-expressing cell lines were we able to detect increased phosphorylation of PERK or eIF2 α (Figure 1A). Consistent with this, we did not measure altered glutathione levels in Ero1-overexpressing cells (see below, Figure 4D). As the splicing of XBP1 mRNA, another hallmark of ER stress signalling, was only weakly observed (Figure 1A), the overexpression of Ero1 in our cell lines just slightly triggered ER stress. Thus, the doxycyclin-inducible system provided a good tool to analyse the effects of Ero1 overexpression.

Similar to transient expression of Ero1 in HeLa cells (Benham *et al*, 2000; Bertoli *et al*, 2004; Dias-Gunasekara

et al, 2005), the stably transfected Ero1 constructs showed characteristic gel mobilities when analysed under non-reducing conditions (Figure 1B). The wild-type Ero1 α and Ero1 α -C394A proteins were predominantly confined to the OX2 form (Figure 1B). This was unlike the case for endogenous Ero1 α where the relative abundance of Ero1 α redox forms varied significantly, or HeLa cells where a considerable fraction of overexpressed Ero1 α was found in the OX1 configuration (Supplementary Figure S2; Anelli *et al*, 2002; Bertoli *et al*, 2004). As observed previously (Bertoli *et al*, 2004), Ero1 α -C131A was unable to form OX2 and migrated as OX1. In addition to these monomeric forms, all four exogenous Ero1 proteins were found in a mixed-disulphide complex with endogenous PDI (Figure 1B). Importantly, the Ero1 variants clearly demonstrated oxidase activity as judged by their ability to increase the oxidation level of both active sites in PDI (Figure 1C). In a mock cell line stably transfected with empty vector, the redox state of PDI was not affected by doxycyclin treatment (data not shown). The finding that PDI became oxidized by the presumed dominant-negative Ero1 α -C394A was surprising. Furthermore, overexpression of Ero1 α -C394A showed the same effect as wild-type Ero1 α on the redox state of endogenous Ero1 α (see below, Figure 3A). These data indicated that Ero1 α -C394A does not exert an effect through a dominant-negative, but rather a competitive mechanism (see Supplementary data).

A disulphide bond between Cys⁹⁴ and Cys¹³¹ defines the OX2 configuration of Ero1 α

To explore the molecular basis for the OX1–OX2 interconversion in Ero1 α , we set out to determine the disulphide-bridge pattern of the protein. We first treated doxycyclin-induced Ero1 α cells with the cell-permeable alkylating agent *N*-ethylmaleimide (NEM) to prevent post-lysis disulphide exchange and then purified His-tagged Ero1 α under denaturing conditions. Consistent with the results in Figure 1B, overexpressed Ero1 α was trapped predominantly in the OX2 form. Purified Ero1 α OX2—some of which had been obtained from cells labelled with ³⁵S-Cys (Figure 2A)—was digested with trypsin, and the resulting peptides were separated by reverse-phase HPLC. Analysis by mass spectrometry (MS) of the HPLC fractions A, B and C containing ³⁵S-labelled material allowed the identification of disulphides in Ero1 α OX2 (Figure 2B and Table I).

Reduction and alkylation of the peptides in fraction B revealed peaks with masses corresponding to the peptides Arg⁸³–Arg^{96/97} (containing Cys⁸⁵ and Cys⁹⁴), Ile³⁸⁸–Arg³⁹⁸ (containing Cys³⁹¹, Cys³⁹⁴ and Cys³⁹⁷) and Tyr¹²⁰–Arg¹³⁶ (containing Cys¹³¹) (Figure 2D), which were not detected in the non-reduced fraction (Figure 2C). This suggested the existence of a disulphide-linked tri-peptide. Indeed, further digestion of the non-reduced fraction with endopeptidase AspN, which separated Cys⁸⁵ and Cys⁹⁴ by cleaving at Asp⁹⁰, followed by MS confirmed this notion (Table I). Specifically, under these conditions, we detected three peaks (Figure 2E) that demonstrated the presence of disulphides between the peptides comprising Cys⁸⁵ and Cys³⁹¹/Cys³⁹⁴/Cys³⁹⁷, and Cys⁹⁴ and Cys¹³¹, respectively (Figure 2B). In agreement with the presence of the Cys⁹⁴–Cys¹³¹ disulphide in Ero1 α OX2, both Ero1 α mutants C131A (Figure 1B) and C94S are unable to form OX2 (Bertoli *et al*, 2004). By comparison with Ero1p where one regulatory disulphide is

Figure 1 Characterization of inducible Ero1 cell lines. (A) Ero1 α (α), Ero1 α -C131A (C131A), Ero1 α -C394A (C394A) and Ero1 β (β) cells were induced with doxycyclin (Dox) for 0, 24 or 48 h, the cell lysates normalized for protein content, separated by SDS-PAGE under reducing conditions and analysed by western blotting using the indicated antibodies. The distribution of spliced and unspliced XBP1 mRNA was assessed by RT-PCR and analysed on an agarose gel. As positive and negative controls for the phosphorylation of PERK and eIF2 α , and the splicing of XBP1 mRNA, HEK293 cells (HEK) were treated or not with the known ER stress inducer DTT (2 mM, 1 h). (B) Ero1 cells were incubated with or without doxycyclin (Dox), treated with NEM to quench post-lysis thiol-disulphide exchange reactions, and the oxidation state of exogenous Ero1 variants was visualized by western blotting using α His after non-reducing SDS-PAGE. The gel mobilities of monomeric Ero1 α variants and Ero1 β are indicated as OX1, OX2 and β OX, respectively. α + PDI and β + PDI denote the mixed disulphides between Ero1 α /Ero1 β and PDI. Note that compared with the endogenous protein (see, e.g., Figure 3A), the epitope-tagged Ero1 α proteins migrated slower by SDS-PAGE. Asterisk, background band; X, His-tagged 75 kDa molecular weight marker (leakage from neighbouring lane); filled circle, unidentified mixed-disulphide complex preferentially formed with Ero1 α -C131A (our unpublished data). (C) The *in vivo* redox state of the two active sites in PDI (a and a') in uninduced (-Dox) versus doxycyclin-induced (+Dox) Ero1 cells (α , β , C394A, C131A) was determined as described previously (Appenzeller-Herzog and Ellgaard, 2008a). After *in situ* modification of free cysteines with NEM, metabolically labelled PDI was immunoprecipitated, and disulphide-bonded cysteines were reduced and then modified with mPEG-mal. Differentially alkylated PDI was analysed by SDS-PAGE and phosphorimaging. Samples reduced with DTT and TCEP, or oxidized with diamide ahead of NEM-modification, served as mobility markers. Red, both active sites reduced; Ox, both active sites oxidized; Semi-ox, semi-oxidized PDI (owing to the faint appearance of the two semi-oxidized forms mainly present in the uninduced lanes, arrowheads mark their mobility in the autoradiograms). Note that mPEG-mal-modified bands are not sharp in appearance because of the large size dispersity of the reagent.

formed by Cys⁹⁰ and Cys³⁴⁹ (Figure 2B), the long-range disulphide bridge involving Cys⁸⁵ was most likely to Cys³⁹¹, one of three cysteines in the Ile³⁸⁸-Arg³⁹⁸ peptide. The internal disulphide detected in this peptide (Table I) would then be between Cys³⁹⁴ and Cys³⁹⁷ and represent the oxidized state of the inner active site. Additional disulphides were identified between Cys³⁵ and Cys⁴⁶ or Cys⁴⁸, Cys³⁷ and Cys⁴⁶ or Cys⁴⁸, and Cys²⁰⁸ and Cys²⁴¹ (fractions A + C, Table I). Finally, the peptide comprising Cys¹⁶⁶ was not detected in an NEM-modified state, but only upon reduction and alkylation of fraction C (Table I). Although this suggested it to be disulphide-bonded, we did not identify a potential partner. No additional Cys-containing Ero1 α peptides were detected in the MS analysis, suggesting that the disulphide pattern depicted in Figure 2B represents the situation in the large majority of protein molecules.

Cys¹³¹ competes with PDI for disulphide exchange with Cys⁹⁴-Cys⁹⁹

Ero1-catalysed oxidation of PDI is initiated through the nucleophilic attack by a deprotonated active-site cysteine in PDI on the disulphide bond between the Ero1 shuttle cysteines (Cys⁹⁴-Cys⁹⁹ in Ero1 α ; Figure 2B). Consequently, a transient mixed-disulphide intermediate is formed between PDI and Cys⁹⁴. The present identification of the intramolecular Cys⁹⁴-Cys¹³¹ disulphide bond that defines Ero1 α OX2 suggested an alternative reaction pathway. Here, a Cys¹³¹-derived thiolate anion would react with the Cys⁹⁴-Cys⁹⁹ active-site disulphide to form Cys⁹⁴-Cys¹³¹ and thus inactivate the enzyme.

To investigate the hypothesis that the conserved Cys¹³¹ (Supplementary Figure S3) competes with reduced PDI, we examined if manipulation of substrate availability influenced

Figure 2 The OX2 redox species of Ero1 α is molecularly defined by the regulatory Cys⁹⁴–Cys¹³¹ disulphide. **(A)** Ero1 α myc6His was two-step purified from unlabelled or ^{35}S -Cys-labelled, doxycyclin-induced and NEM-treated Ero1 α cells. A Coomassie-stained gel (Coom.) and a phosphorimager scan (^{35}S) from the unlabelled and labelled protein batch, respectively, are shown. Ero1 α OX2 was shifted to the reduced (Red) form upon treatment of the sample with DTT. α + PDI, mixed-disulphide complex between Ero1 α and PDI; asterisks, background bands. **(B)** Schematic representation of the cysteine connectivities in oxidized (ox.) Ero1p (Gross *et al*, 2004) and Ero1 α OX2 (this study). The cysteines are shown as yellow, green (outer active site) or blue (inner active site) circles with amino-acid numbering, and disulphides as thick grey (likely structural), black (active site) or red (reported or inferred regulatory function; Sevier *et al*, 2007 and this study) lines. The thick orange line at Cys¹⁶⁶ indicates the connection to a likely (but unidentified) disulphide partner. The question marks denote cysteines with unresolved oxidation state. The flexible loop regions are coloured in light blue. The braces for Cys^{35/37} and Cys^{46/48} indicate the two possible connections that could not be distinguished from our data. The three disulphide-linked peptides after trypsin cleavage and their trimming products upon further digestion with AspN are shown in grey boxes where the solid and dashed borders reflect their connectivity. Note that the alternative tryptic cleavage at Arg⁹⁶ or Arg⁹⁷ gave rise to two peptides that were both disulphide-linked to the Tyr¹²⁰–Arg¹³⁶ peptide (see panel E). **(C, D)** Mass spectra of HPLC fraction B before (C) and after (D) reduction and alkylation with DTE and IAA ($\pm$ DTE $\pm$ IAA), respectively. The monoisotopic masses at m/z 1445.62 (insets), 1792.81, 1948.90 and 2083.92 appear only in the reduced and alkylated sample and correspond to the four IAA-modified peptides labelled in the spectrum (see also Table I). **(E)** The monoisotopic masses at m/z 1944.88, 2802.83 and 2958.34 upon AspN digestion of fraction B correspond to the indicated disulphide-linked di-peptides (see also Table I). Bordering patterns of boxes match those in panel B.

the relative levels of OX1 and OX2. First, we reasoned that overexpression of Ero1 would decrease the abundance of reduced PDI (see Figure 1C) and thereby lower the substrate

level for endogenous Ero1 α . We therefore studied the redox state of endogenous Ero1 α upon overexpression of exogenous Ero1. In this experiment, exogenous Ero1 α variants were

Table 1 Mapping of disulphide bonds in Ero1 α

Fractions ^a	Treatments ^b		Peptides ^c	Cysteines ^d	Observed mass ^e (MH ⁺)	Mass difference ^f (MH ⁺)
	DTE + IAA	Second digest				
A	–	–	Cys ³⁵ –Arg ⁵⁵	35, 37, 46, 48	2380.99	–3.95 (2 S-S)
	+	–	Cys ³⁵ –Arg ⁵⁵	35, 37, 46, 48	2613.10	228.16 (4 IAA)
	–	AspN	Cys ³⁵ –Asp ⁴⁴ + Asp ⁴⁵ –Cys ⁴⁸	35, 37, 46, 48	1570.54	–4.01 (2 S-S)
B	+	–	Arg ⁸³ –Arg ⁹⁶	85, 94	1792.81	114.06 (2 IAA)
			Arg ⁸³ –Arg ⁹⁷	85, 94	1948.90	114.05 (2 IAA)
			Tyr ¹²⁰ –Arg ¹³⁶	131	2083.92	57.06 (1 IAA)
			Ile ³⁸⁸ –Arg ³⁹⁸	391, 394, 397	1445.62	171.08 (3 IAA)
	–	AspN	Arg ⁸³ –Asn ⁸⁹ + Asp ³⁹⁰ –Arg ³⁹⁸	85, 391, 394, 397	1944.88	–3.96 (2 S-S)
			Asp ⁹⁰ –Arg ⁹⁶ + Tyr ¹²⁰ –Arg ¹³⁶	94, 131	2802.23	–1.97 (1 S-S)
			Asp ⁹⁰ –Arg ⁹⁷ + Tyr ¹²⁰ –Arg ¹³⁶	94, 131	2958.34	–1.97 (1 S-S)
C	+	–	His ¹⁵⁸ –Arg ¹⁸⁷	166	3493.53	57.06 (1 IAA)
			Ile ¹⁹⁹ –Lys ²¹⁵	208	2182.12	57.05 (1 IAA)
			Arg ²¹⁶ –Lys ²⁴⁴	241	3282.52	56.98 (1 IAA)
			Arg ²¹⁶ –Arg ²⁴⁵	241	3438.50	56.85 (1 IAA)
	–	GluC	Ala ¹⁷⁶ –Arg ¹⁸⁷		1431.74	
			Tyr ¹⁷⁸ –Arg ¹⁸⁷		1231.68	
			Ile ¹⁹⁹ –Glu ²⁰⁶		1065.48	
			Arg ²¹⁶ –Glu ²³⁰		1555.70	
			Asn ²³¹ –Glu ²³⁸		1059.48	
			Asn ²⁰⁷ –Lys ²¹⁵ + Gly ²³⁹ –Glu ²⁴³	208, 241	1595.75	–2.05 (1 S-S)

^aFractions from the tryptic digest of Ero1 α identified to contain disulphide-linked peptides.

^bThe fractions were analysed by MS before and after reduction with DTE followed by alkylation with IAA. In some cases, the samples were further digested with endoproteinase AspN or endoproteinase GluC.

^cPeptides identified by MALDI-TOF-MS.

^dPositions of cysteines contained in the identified peptides.

^eObserved monoisotopic molecular masses determined by MALDI-TOF-MS.

^fDifference between observed and calculated masses. The number and type of modifications corresponding to the mass difference are given in parentheses: disulphide bridge (S-S) and alkylation with iodoacetamide (IAA).

depleted through the His-tag after cell lysis (Supplementary Figure S4), allowing detection of the endogenous protein only by subsequent western blotting. Indeed, the mixed-disulphide complex between endogenous Ero1 α and PDI became less abundant when Ero1 was overexpressed (Figure 3A). Concomitantly, we observed a moderate, but clear and reproducible (see Supplementary Figure S5) decrease in the OX1/OX2 ratio of endogenous Ero1 α . These effects were seen for all four constructs. Importantly, the OX2 shifts observed upon overexpression of Ero1 variants were not a result of general ER hyperoxidation, as the overexpression of Ero1 α did not perturb the GSSG/GSH ratio (see below, Figure 4).

We next used two different HeLa-derived cell lines stably transfected with a PDI-specific short hairpin RNA (shRNA) (Ou and Silver, 2006) to study a potential influence of PDI knockdown on the redox state of Ero1 α (Figure 3B). Under these conditions of decreased substrate level, the OX1/OX2 ratio of endogenous Ero1 α was clearly diminished and less covalent Ero1 α –PDI complex was present (Figure 3C). Importantly, cells treated with siRNA against Erp57, a close homologue of PDI, or a transmembrane PDI-family member called TMX3 (Haugstetter *et al*, 2005) did not exhibit an OX2 shift in Ero1 α (Figure 3D).

In contrast to the above-mentioned data, overexpression of PDI (but not of Erp57 or TMX3) affected the activation state of endogenous Ero1 α so that the OX1/OX2 ratio was now increased (Figure 3E). The redox state of exogenous Ero1 α responded to co-expression of PDI in a similar way (Otsu *et al*, 2006; and data not shown). We concluded that the Cys¹³¹-linked activation state of Ero1 α is governed by the availability of its own substrate, PDI.

The OX2 switch in Ero1 α contributes to ER redox homeostasis

We next characterized the OX2-impaired C131A mutant of Ero1 α . Yeast cells expressing the Ero1p-C150A-C295A mutant that lacks one regulatory disulphide and also does not form the other under normal cellular conditions exhibit a severe growth defect caused by Ero1p hyperactivity (Sevier *et al*, 2007). In contrast, induced and uninduced Ero1 α -C131A cells proliferated equally well (our unpublished observation). Treatment of yeast cells with L-buthionine-sulfoximine (BSO), an inhibitor of GSH synthesis, leads to oxidative inactivation of Ero1p-C150A-C295A through the formation of the second regulatory disulphide Cys⁹⁰–Cys³⁴⁹ (corresponding to Cys⁸⁵–Cys³⁹¹ in Ero1 α) (Sevier *et al*, 2007). However, Ero1 α -C131A in HEK293 cells remained in the partially oxidized OX1 configuration irrespective of BSO-mediated depletion of the cellular GSH pool (Figure 4A). These results suggested that in Ero1 α -C131A, unlike the case in Ero1p-C150A-C295A, Cys⁸⁵–Cys³⁹¹ remained oxidized under steady-state ER redox conditions.

To more closely monitor the effect of exogenous Ero1 variants on ER redox homeostasis, we assayed the redox distribution of ER-resident oxidoreductases and GSH in Ero1-expressing cells. We first examined the *in vivo* redox state of the PDI homologues Erp57 and TMX3 by means of a 4-acetamido-4'-maleimidylstilbene-2,2'-disulphonic acid (AMS) alkylation assay that results in a decreased electrophoretic mobility of oxidized species (Supplementary Figure S6). Overexpression of Ero1 α did not cause any clear effect on the redox state of the two oxidoreductases (Figure 4B; top panel, compare lanes 1 and 2, and 5 and 6). Interestingly,

Figure 3 The redox state of Cys⁹⁴-Cys¹³¹ is modulated by the availability of PDI. **(A)** Uninduced (–Dox) or induced (+Dox) Ero1 cells (α , C131A, C394A, β) were treated with NEM, solubilized in lysis buffer and the lysates were depleted of exogenous, His-tagged Ero1 α using TALON beads. The supernatant was subjected to precipitation with ConA-sepharose, and the glycoprotein fraction was analysed by western blotting using α Ero1 α following non-reducing SDS–PAGE. Stripping and reprobing the same membrane with α PDI identified the ~130 kDa band as the mixed-disulphide complex between endogenous Ero1 α and PDI (α +PDI, data not shown). **(B)** Lysates of a control (Ctrl) shRNA clone (5-1) and two PDI knockdown (kd) clones (4-1 and 1-2) (Ou and Silver, 2006) were analysed by western blotting using α PDI to monitor knockdown efficiency and α -Actin as loading control. **(C)** 5-1, 4-1 and 1-2 cells were treated with NEM, lysed and the ConA-sepharose-precipitated glycoprotein fraction was subjected to α Ero1 α western blot analysis. The three independent samples were not normalized for protein content, meaning that no conclusions about the relative abundance of Ero1 α between lanes can be made. **(D)** HeLa cells left untreated or treated with siRNAs against TMX3 (siTMX3) or ERp57 (siERp57) were harvested and lysed in the presence of NEM. Lysates were directly analysed either by western blotting with the indicated antibodies or, for the detection of Ero1 α , after ConA-precipitation to concentrate the protein. Similar results were obtained with HEK293 cells (not shown). The hairline indicates where a lane has been removed. **(E)** HEK293 cells were transfected with the indicated cDNAs, harvested and lysed in the presence of NEM. Lysates were either directly subjected to western blot analysis using α PDI and α HA (lower panels) or incubated with ConA-sepharose to concentrate the glycoprotein fraction ahead of western blot detection of endogenous Ero1 α (upper panel).

however, overexpression of Ero1 α -C131A or Ero1 β , which also exhibits only one oxidized species (Dias-Gunasekara *et al*, 2005), caused a moderate oxidative shift in the redox state of both TMX3 and ERp57 (Figure 4B; middle and bottom panel, compare lanes 1 and 2, and lanes 5 and 6). As documented in the Supplementary data, for ERp57, the AMS assay most clearly monitors the redox state of the a' domain (Supplementary Figure S6). Moreover, depletion of glutathione by treatment with BSO aggravated the oxidative phenotypes of Ero1 α -C131A- and Ero1 β -overexpressing cells (Figure 4B; middle and bottom panel, compare lanes 2 and 4, and 6 and 8). Thus, *in vivo*, the activity of Ero1 α is counterbalanced by the glutathione redox buffer and negatively regulated by Cys¹³¹-mediated inactivation.

The oxidative effect on TMX3 and ERp57 caused by overexpression of Ero1 α -C131A and Ero1 β may have resulted from moderate, direct oxidation of these PDI-like proteins and/or from oxidation through the glutathione redox buffer. To test if overexpression of Ero1 variants affected the overall

ER thiol–disulphide homeostasis, we determined the ratio of oxidized to reduced glutathione (GSSG/GSH) in Ero1-expressing and control cells. As GSSG is hardly present in the cytosol, mitochondria and nuclei (Go and Jones, 2008), and as the ER by far is the largest organelle of the secretory pathway in mammalian cells (De Menezes *et al*, 1996), it can be assumed that most GSSG detected in an assay on whole cells is present in the ER. Treatment of Ero1 α -C131A and Ero1 β cells with doxycyclin significantly increased GSSG/GSH, whereas ectopic expression of wild-type Ero1 α had no effect (Figure 4C and Supplementary Figure S7). These differences could potentially be a result of Ero1 construct-specific changes in the total glutathione concentration (GSSG + GSH). Although doxycyclin treatment *per se* produced a marginal increase in GSSG + GSH (Figure 4D, mock cells), none of the Ero1 proteins further modulated cellular glutathione levels when overexpressed (Figure 4D). These results differ from the situation in transiently transfected HeLa cells (Molteni *et al*, 2004). The effects on GSSG/GSH

Figure 4 Increased *in vivo* activity of Ero1 α -C131A and Ero1 β is buffered by GSH. **(A)** Ero1 α and C131A cells were induced (+Dox), treated with or without BSO and processed in the presence of NEM before α His western blot analysis. **(B)** Ero1 cells were treated with doxycyclin (Dox) and/or BSO as indicated, and the *in vivo* redox states of TMX3 and Erp57 trapped by differential alkylation with NEM and AMS, followed by western blotting. Treatment with BSO reduced GSSG + GSH to 20% of control (data not shown). The mobilities of the reduced (Red) and oxidized (Ox) species, as verified by control samples using lysates from DTT- or diamide-treated cells (not shown), are indicated. Note that the mobility shift of the Erp57 band reflects the oxidation state of the α' domain (Supplementary Figure S6). The results are representative of three independent experiments. **(C)** Cellular GSSG levels increase upon overexpression of Ero1 α -C131A or Ero1 β . Ero1 cells (α , C131A, β) were left untreated or induced with doxycyclin for 24 h and analysed for the intracellular levels of GSSG and GSH. The relative changes of GSSG/GSH caused by Ero1 overexpression along with 95% confidence intervals as calculated by using a linear model to correct for variation between experimental batches are plotted ($n=12$; for the raw data, refer to Supplementary Figure S7). Asterisks indicate statistical significance ($P<0.03$). n.s., not significant. **(D)** GSSG + GSH and total protein was determined in samples obtained from the indicated cell lines with (+Dox) or without (-Dox) doxycyclin induction for 24 h. GSSG + GSH (in pmol) was normalized to protein content (in μ g) (mean $\pm$ s.d., $n=3$).

suggested that the Ero1-dependent oxidative shifts of TMX3 and Erp57 were at least in part caused by a change in the overall ER thiol-disulphide homeostasis. Taken together, the overexpression of Ero1 variants lacking an inactivated OX2 form affected the glutathione-buffered ER redox homeostasis in the direction of more oxidizing conditions.

Discussion

A central feedback loop for redox control in the ER of human cells

The results presented here provide critical mechanistic insight into ER redox control in human cells. The most oxidized redox form of Ero1 α , OX2, represents a negatively regulated species. The silencing mechanism is straightforward and direct—the formation of the Cys⁹⁴-Cys¹³¹ regulatory disulphide bridge covalently blocks the catalytically essential, first shuttle cysteine, Cys⁹⁴. Owing to competition between Cys¹³¹ and substrate thiols for reaction with the active-site Cys⁹⁴-Cys⁹⁹ disulphide, the availability of substrate would predictably regulate the formation of Cys⁹⁴-Cys¹³¹. Indeed, the manipulation of both the redox state (Figure 3A) and levels (Figure 3C and E) of the Ero1 α substrate PDI affected the extent of Cys¹³¹-mediated shutdown that is given by the OX1/OX2 ratio.

Protein disulphide isomerase fulfils a central position in ER thiol-disulphide homeostasis by passing on Ero1-generated oxidizing equivalents to the ER thiol pool. Our data suggest that Ero1 α is designed to adapt its activation state to the redox distribution of PDI through a direct feedback loop

(Figure 5). Consequently, Ero1 α -mediated generation of disulphides will be stalled by feedback inhibition when, under oxidizing ER conditions, PDI accumulates in the oxidized form. Under reducing conditions, however, PDI active-site cysteines in the reduced state will be abundant as substrates for Ero1 α . By interacting with Ero1 α in the OX1 state to reduce Cys⁹⁴-Cys⁹⁹, reduced PDI will disfavour Cys¹³¹-mediated shutdown. At the same time, overexpression of a PDI variant devoid of all four active-site cysteines also leads to some OX2-to-OX1 conversion in Ero1 α (Otsu *et al.*, 2006), indicating that this mutant binds and stabilizes OX1 as well. We suggest that the PDI-Ero1 α feedback loop relies on both thiol-disulphide exchange and a specific binding of PDI to Ero1 α . Accordingly, overexpression or downregulation of the PDI-related oxidoreductases TMX3 and Erp57 did not influence the OX1/OX2 ratio in Ero1 α .

In most experiments, we observed OX1/OX2 ratios lower than one (Figure 3A, C and E and Supplementary Figure S2, lower panel). However, it is worth noting that the *in vivo* redox distribution of endogenous Ero1 α is variable under normal cellular conditions, presumably reflecting physiological fluctuations in the thiol-disulphide household of the ER (Supplementary Figure S2). Still, in all our experiments and regardless of the thiol-trapping method used (NEM or rapid acidification by trichloroacetic acid), endogenous Ero1 α -OX2 was prominently detected. Mammalian cells apparently maintain a considerable pool of inactive molecules that can promptly be activated upon need. Indeed, ER reoxidation after DTT treatment of cells is in the time scale of seconds and

Figure 5 Model for homeostatic control of the PDI redox state by Cys¹³¹-dependent feedback regulation of Ero1 α . The thiol–disulphide composition in the ER depends on the load of nascent polypeptides—many of which are substrates for PDI-mediated oxidation—as well as on the redox distribution of ER-localized glutathione. As both of these terms vary under physiological conditions, the redox state of the thiol–disulphide system in the ER can fluctuate (reducing versus oxidizing ER conditions). As detailed in the text, such fluctuations are likely mirrored in the steady-state distribution between oxidized and reduced PDI (grey box). Panels (A) and (B) depict the two extreme cases where the outer active-site disulphide in Ero1 α is exclusively attacked by a PDI- or Cys¹³¹-derived thiolate anion. In the cell, these two reaction pathways compete with each other. (A) Under reducing ER conditions, PDI (for simplicity, only one active site in PDI is depicted) accumulates in the reduced form. Reduced PDI is a substrate for oxidation by Ero1 α , which is initiated through the intermolecular nucleophilic attack by an active-site cysteine in PDI on Cys⁹⁴ in the oxidized outer active site of Ero1 α . The competing intramolecular reaction of Cys¹³¹ with the Cys⁹⁴–Cys⁹⁹ disulphide is disfavoured by the abundance of reduced PDI. A thiol–disulphide exchange reaction between Ero1 α (in the active form) and PDI then results in the formation of oxidized PDI. By this mechanism, the activity of Ero1 α counterbalances reducing ER conditions. (B) Under oxidizing ER conditions, PDI is predominantly oxidized and the outer active-site disulphide of Ero1 α will, therefore, preferentially react with Cys¹³¹. The resulting OX2 configuration of Ero1 α is inactive because the outer active Cys⁹⁴ is covalently blocked by Cys¹³¹. The mechanism of reactivation of Ero1 α OX2 remains unclear. In principle, it could involve a nucleophilic attack of Cys⁹⁹ on Cys⁹⁴ to resolve Cys⁹⁴–Cys¹³¹, but the oxidation state of Cys⁹⁹ in OX2 is not known (indicated by the question mark).

further accelerated by overexpression of Ero1 α (our unpublished observations).

An intact OX2 switch is required for ER redox homeostasis

Our results showed that despite the higher relative abundance of oxidized PDI upon overexpression of Ero1 α (Figure 1C), the general ER redox state did not measurably change (Figure 4B and C). This homeostatic stability depended on an intact OX2 switch in overexpressed Ero1 α that was absent in Ero1 α -C131A, and apparently also Ero1 β (Figure 4B and C). The latter finding fits well with the observation that Ero1 β does not form a redox form similar to OX2 (Dias-Gunasekara *et al*, 2005), although a cysteine at the position equivalent of Ero1 α Cys¹³¹ is conserved in Ero1 β . In contrast to Ero1 α , however, Ero1 β harbours three *N*-glycosylation sites proximal to that cysteine, two of which have been experimentally verified (Lewandrowski *et al*, 2006). Although speculative at present, it is possible that a conformation similar to OX2 in Ero1 β is sterically disfavoured by these *N*-glycans. Ero1 β also harbours an

additional cysteine that is not conserved in Ero1 α . Overall, a possible disulphide-based regulatory mechanism of Ero1 β activity awaits further clarification.

The mechanism by which overexpression of Ero1 α -C131A and Ero1 β perturb the ER redox balance is unclear. However, the effect may arise from faster oxidation kinetics and an increased rate of disulphide generation that is not visible in the steady-state readout of the PDI redox state where these two proteins showed an effect similar to Ero1 α (Figure 1C). In support of this, the deregulated Ero1 α -C150A-C295A mutant shows increased reaction kinetics *in vitro* (Sevier *et al*, 2007).

Glutathione counterbalances the activity of Ero1

Apart from the OX2 regulatory feedback mechanism, it has previously been shown that a proper ER redox balance critically depends on GSH (Cuozzo and Kaiser, 1999; Chakravarthi and Bulleid, 2004; Molteni *et al*, 2004). Accordingly, the redox states of Erp57 and TMX3 showed more prominent oxidative shifts upon Ero1 α -C131A or Ero1 β overexpression when cellular glutathione levels were diminished by treatment with BSO (Figure 4B). Although we

cannot exclude the possibility that TMX3 and/or ERp57 were also directly oxidized by Ero1 α -C131A and Ero1 β , the Ero1-dependent oxidative shifts in these two proteins were clearly less prominent than those observed in PDI (Figure 1C). Therefore, the shifts in TMX3 and ERp57 are probably mainly promoted by the increase in GSSG/GSH (Figure 4C). Unfortunately, our assay did not allow us to accurately determine Ero1-dependent changes in GSSG/GSH at the low levels of glutathione present in BSO-treated cells.

Unique structural and functional features of Ero1 α OX2

The MS analysis of Ero1 α OX2 revealed several distinguishing features compared with the oxidized form of Ero1p. Most intriguingly, a disulphide corresponding to the long-range regulatory Cys¹⁵⁰–Cys²⁹⁵ in Ero1p, which connects the extended loop with the core, is missing in Ero1 α OX2 that instead contains the Cys⁹⁴–Cys¹³¹ regulatory disulphide (Figure 2B). This is positioned in the extended loop, with Cys¹³¹ reaching directly into the outer active site. As a consequence, the regulatory mechanisms are clearly different between the two enzymes. Structurally, the constraint placed by the Cys⁹⁴–Cys¹³¹ disulphide on the backbone conformation of the extended loop in Ero1 α must cause the structure of this region to differ significantly from the one in oxidized Ero1p.

The position of the second regulatory disulphide in Ero1p is conserved in Ero1 α (Cys⁸⁵–Cys³⁹¹; Figure 2B), but it does not appear to be equally regulated—in Ero1 α -C131A, unlike the case in Ero1p-C150A-C295A (Sevier *et al*, 2007), the disulphide resisted GSH-mediated reduction *in vivo*. On the basis of the sequence similarity, we consider it most likely that Cys⁸⁵–Cys³⁹¹ constitutes a regulatory disulphide in Ero1 α as well that, however, seems to be more stable in the ER than its counterpart in Ero1p. Unfortunately, the potential functional conservation of Cys⁸⁵–Cys³⁹¹ will be difficult to test *in vivo*, as both Ero1 α -C85S and Ero1 α -C391A—presumably owing to folding defects when expressed in cultured cells—display reduced oxidase activity, a gel migration pattern different from wild-type Ero1 α , and the formation of high-molecular weight complexes with PDI unlike the normal one at ~130 kDa (Bertoli *et al*, 2004).

The data identified three additional disulphides. Two connect the two N-terminal Cys-Xaa-Cys clusters—as an N-terminally truncated version of Ero1p was used for structural studies, the oxidation state of the Cys⁴⁰/Cys⁵² couple in Ero1p is not known (Figure 2B). On the basis of sequence alignment and homology modelling, the Cys²⁰⁸–Cys²⁴¹ bond is expected to be structural and connect two antiparallel helices of the core structure. Although Cys²⁰⁸ is conserved, the equivalent bond is missing in Ero1p that does not contain a cysteine corresponding to Cys²⁴¹. We did not recover the tryptic peptide containing Cys⁹⁹ and Cys¹⁰⁴. It is tempting to speculate, however, that in Ero1 α OX2 where Cys¹³¹ blocks Cys⁹⁴ (the partner of Cys⁹⁹ in the active enzyme), Cys⁹⁹ forms a disulphide with the close-by Cys¹⁰⁴. However, at present, we cannot rule out that either Cys⁹⁹ or Cys¹⁰⁴ is connected to Cys¹⁶⁶, the presumed disulphide partner of which was not detected in our MS analysis.

Conclusion

On the basis of the identification of a crucial regulatory disulphide bond in human Ero1 α , we show the existence of

a feedback mechanism where the activation state of this ER oxidase is controlled by the redox state and availability of its substrate, PDI. This places PDI as a central regulator of ER luminal redox homeostasis in human cells.

A principle conclusion of this study is that human Ero1 α differs significantly from Ero1p. In addition, the redox distribution of PDI is significantly more oxidizing in yeast than in HEK293 cells (Frandsen and Kaiser, 2000; Appenzeller-Herzog and Ellgaard, 2008a). Despite these differences, it is quite possible, on the basis of the conservation of Ero1 and PDI, that yeast PDI has an important function in regulating the redox state of Ero1p. These details of the yeast system are, however, presently unknown. Clearly, more experiments are required to clarify the exact mechanisms that govern the activation of Ero1p, Ero1 α and Ero1 β , as well as redox differences between various cell types. Moreover, in mammalian cells, other redox enzymes such as the QSOX oxidases (Thorpe and Coppock, 2007) or ER-resident PDI peroxidases (L Ruddock, personal communication) may have important, as yet unrecognized functions in ER redox homeostasis.

It is interesting to consider how the ER redox system has evolved to deal with the inherent danger of hyperoxidation. *In vitro*, PDI is a relatively poor substrate of Ero1p compared with the much more reducing thioredoxin (Sevier *et al*, 2007). It thus appears that the ER redox system has evolved to carefully regulate redox conditions at the expense of optimal catalytic activity.

Materials and methods

Recombinant DNA

DNA work was performed as detailed in the Supplementary data. pcDNA3/PDI was a gift from I Braakman, Utrecht, pcDNA3/HA-ERp57 from R Sitia, Milan, and pcDNA3/TMX3-HA has been described previously (Haugstetter *et al*, 2005).

Cell lines, transfection and antibodies

HEK293 and HeLa cells were grown in α -minimal essential medium (Invitrogen), supplemented with 10% fetal calf serum at 37°C, 5% CO₂. Doxycyclin-inducible cell lines were generated by transfecting pcDNA5/Ero1 α myc6his, pcDNA5/Ero1 α -C131Amyc6his, pcDNA5/Ero1 α -C394Amyc6his and pcDNA5/Ero1 β myc6his into Flp-In TRex-293 cells (Invitrogen) using the CaPO₄ method and selecting for stably transfected clones with 100 μ g/ml hygromycin B and 15 μ g/ml blasticidin (both Invitrogen). Ero1-expressing cells were grown in the presence of these antibiotics. Ero1 expression was induced for 24 h (unless otherwise stated) using 1 μ g/ml doxycyclin (Sigma). BSO (1 mM) was added for 20 h. PDI shRNA clones 5-1, 4-1 and 1-2 (Ou and Silver, 2006), a gift from W Ou, Bethesda, were maintained in the presence of 100 μ g/ml hygromycin B. Transient transfections were performed with Lipofectamine 2000 (Invitrogen). For the knockdown of ERp57 and TMX3, cells were transfected with the Hs_GRP58_5 HP (10 nM) and Hs_TXNDC10_7 HP (20 nM) siRNAs (both from Qiagen) using Lipofectamine RNAiMAX (Invitrogen) and analysed 72 h post-transfection.

The following mouse monoclonal antibodies were used: TetraHis (α His, Qiagen), HA.11 (α HA, Covance), 9E10 (α myc, Covance), AC-15 (α -actin, Sigma). The rabbit polyclonal antisera used were as follows: D5 (α Ero1 α , a gift from I Braakman, Utrecht), SPA-890 (α PDI, Stressgen), α TMX3 (Haugstetter *et al*, 2005), α ERp57 (a gift from A Helenius, Zürich), α P-PERK (Santa Cruz Biotechnology), α P-eIF2 α and α eIF2 α (both Cell Signaling Technology).

Purification of recombinant Ero1 α

Ero1 α cells grown in twenty 175 cm² T-flasks to ~50% confluency and induced with 1 μ g/ml doxycyclin for 48 h were washed once with ice-cold PBS containing 20 mM NEM and incubated with the same buffer for 20 min at 4°C. Cells were then lysed for 1 h at 4°C in 10 ml lysis buffer (100 mM NaPO₄, pH 8.0, 1% TX-100, 20 mM NEM,

0.2 mM phenylmethylsulfonylfluoride) per T-flask and the lysates cleared by centrifugation (100 000 g, 4°C, 30 min). A total of 50 μ l ConA (Concanavalin A)-Sephacrose 4B (Sigma) was added per 10 ml of lysate, and the bead suspension was incubated for 1 h at 4°C under constant agitation. After precipitation, the beads were washed 4 $\times$ in lysis buffer and 1 $\times$ in the same buffer lacking TX-100. Bound (glyco)proteins were eluted by two consecutive incubations in 5 ml (per 50 μ l ConA beads) denaturation buffer (50 mM NaPO₄, pH 7.0, 300 mM NaCl, 20 mM NEM, 8 M urea) for 1.5 h each at room temperature. The pooled eluate was loaded onto a column packed with 0.5 ml TALON affinity resin (Clontech) and recirculated over the column overnight at room temperature using a peristaltic pump. After washing with 300 bead volumes of denaturation buffer and 20 bead volumes of a 1:1 dilution of the same buffer with H₂O (0.5 $\times$ denaturation buffer), the bound protein was eluted in 3 $\times$ 0.5 ml of 0.5 $\times$ denaturation buffer containing 300 mM imidazole. The purified Ero1 α protein (~50 μ g) was concentrated using a Biomax-5 Ultrafree-0.5 centrifugal filter device (Millipore) and stored at -20°C. In parallel, Ero1 α cells grown in fifteen 10-cm dishes were labelled with 5 mCi ³⁵S-Cys (PerkinElmer) for 24 h in the presence of 1 μ g/ml doxycyclin and 2% fetal calf serum and the protein was purified as described above.

Disulphide-bridge mapping

The samples of labelled (~10⁶ cpm) and unlabelled purified Ero1 α were pooled and digested with sequencing-grade modified trypsin (Promega) at 37°C overnight. Tryptic peptides were separated by reverse-phase HPLC on a μ RPC C₂/C₁₈ PC 2.1/10 column connected to an ÄKTA Explorer (GE Healthcare). The peptides were separated in 0.1% trifluoroacetic acid (TFA) and eluted with a gradient of 80% acetonitrile in 0.1% TFA. Aliquots of each fraction were subjected to liquid scintillation counting to identify ³⁵S-Cys-containing peptides.

For reduction or subdigestion of disulphide bond-containing peptides, 20 μ l of HPLC fractions were dried in a vacuum centrifuge. For reduction, the samples were resuspended in 20 μ l of 15 mM 1,4-dithioerythritol (DTE) in 50 mM ammonium bicarbonate and incubated at 56°C for 45 min. Free cysteines were then alkylated by addition of iodoacetamide (IAA, to 50 mM) and incubation for 30 min in the dark at room temperature. Endoproteinase digestions were performed in 20 μ l of either 50 mM sodium phosphate (pH 8.0) at 37°C for 18 h (AspN, Roche Diagnostics) or 0.1 M ammonium bicarbonate at 37°C for 3.5 h (GluC, Sigma) using 120 ng of each enzyme.

For mass spectrometric analyses using a Voyager DE-PRO MALDI-TOF mass spectrometer (Applied Biosystems), peptide samples were mixed with a saturated solution of 2,5-dihydroxybenzoic acid (LaserBio Labs) in a 1:1 ratio directly on the target probe. All spectra were obtained in positive reflector-ion mode using a nitrogen laser at 337 nm and an acceleration voltage of 20 kV. Typically, 50–100 laser shots were added per spectrum and

calibrated with external standards. The theoretical peptide masses were calculated using the GPMW program (Lighthouse Data).

Sample preparation, AMS and mPEG-mal modification

N-ethylmaleimide-alkylated TX-100 lysates for ConA- or TALON-precipitation were obtained as described for the purification of Ero1 α . Depletion of His-tagged Ero1 was performed by repeated incubation of the lysate with TALON-beads at 4°C on an end-over-end rotator. Reduced and oxidized control lysates were obtained from cells treated with 10 mM DTT or 5 mM diamide (both from Sigma) for 5 min at 37°C in full-growth medium. AMS (Invitrogen) and mPEG-mal (methoxy polyethylene glycol 5000 maleimide, Laysan Bio) modification protocols have been described previously (Appenzeller-Herzog and Ellgaard, 2008a).

Analysis of XBP1 splicing

The splicing of XBP1 mRNA was analysed as described by Yoshida *et al* (2001).

GSH and protein measurements

A total of 5 $\times$ 10⁶ cells per dish were seeded into 10-cm dishes 1 day before treatment with or without doxycyclin for 24 h. Cells were washed twice with PBS and lysed in 400 μ l of 1% sulphasalicylic acid. The lysate was cleared by centrifugation (20 000 g, 5 min) and analysed for GSSG + GSH and GSSG (expressed as GSH equivalents) as described in detail elsewhere (Minich *et al*, 2006). Briefly, GSSG + GSH was measured directly with a 5,5'-dithiobis(2-nitrobenzoic acid)GSH reductase recycling assay, whereas for the determination of GSSG, the lysate was treated with 2-vinylpyridine to modify GSH before analysis. Protein levels were determined using the BCA assay (Pierce).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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