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Identifying Interaction Partners of Yeast Protein Disulfide Isomerases Using a Small Thiol-Reactive Cross-Linker: Implications for Secretory Pathway Proteostasis

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Abstract

Protein disulfide isomerases (PDIs) function in forming the correct disulfide bonds in client proteins, thereby aiding the folding of proteins that enter the secretory pathway. Recently, several PDIs have been identified as targets of organic electrophiles, yet the client proteins of specific PDIs remain largely undefined. Here, we report that PDIs expressed in *Saccharomyces cerevisiae* are targets of divinyl sulfone (DVSF) and other thiol-reactive protein cross-linkers. Using DVSF, we identified the interaction partners that were cross-linked to Pdi1 and Eug1, finding that both proteins form cross-linked complexes with other PDIs, as well as vacuolar hydrolases, proteins involved in cell wall biosynthesis and maintenance, and many ER proteostasis factors involved ER stress signaling and ER-associated protein degradation (ERAD). The latter discovery prompted us to examine the effects of DVSF on ER quality control, where we found that DVSF inhibits degradation of the ERAD substrate CPY*, in addition to covalently modifying Ire1 and blocking activation of the unfolded protein response. Our results reveal that DVSF targets many proteins within the ER proteostasis network and suggest that these proteins may be suitable targets for covalent therapeutic development in the future.

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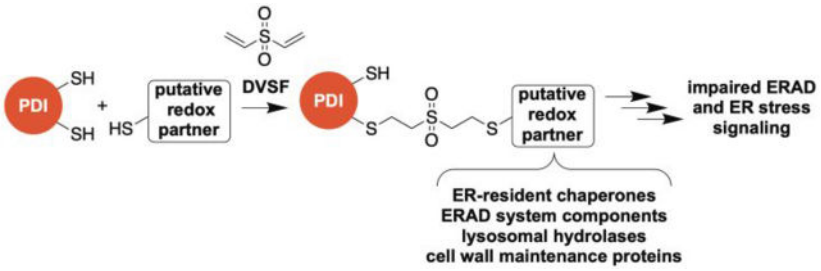

Introduction.

Folding of globular proteins presents a fundamental challenge in biology and involves numerous molecular chaperones that constitute part of the proteostasis network.[1] The proteostasis network also encompasses protein quality control factors that attend to all aspects of protein life cycles in cells, ranging from protein synthesis to post-translational modification and protein metal-binding to degradation.[2] Each subcellular compartment possesses its own array of proteostasis factors, depending on the specific needs of that location. In the oxidizing environment of the eukaryotic ER, the molecular chaperone network consists of members of the Hsp70/Hsp40 system, peptidyl prolyl isomerases, and protein disulfide isomerases (PDIs), all of which carry out specified roles in folding client proteins that enter the secretory pathway.[3, 4]

PDIs constitute one branch of the thioredoxin superfamily, a family of evolutionarily conserved, thiol-dependent enzymes that can facilitate disulfide bond formation and/or rearrangement.[5] In the ER lumen, PDIs aid translocated proteins in forming the correct combination of disulfide bonds as part of their folding process, employing a disulfide exchange relay that is mediated through active site CXXC motifs.[6, 7] PDIs can work in concert with other molecular chaperones that facilitate different aspects of protein folding (e.g., hydrophobic collapse, prolyl isomerization, etc.).[8–11] PDIs also participate in some aspects of ER-associated degradation (ERAD) and ER stress response signaling, indicating wide-ranging functions in secretory pathway proteostasis.[12–17]

Most eukaryotes express multiple PDIs, implying that individual PDIs may interact with different client proteins or have different spatiotemporal or stress-responsive roles.[18] For instance, baker's yeast encodes five different PDIs – Pdi1, Eps1, Eug1, Mpd1, and Mpd2. Of these, only Pdi1 is essential, whereas the other PDIs can rescue *PDI1*-deficient yeast when overexpressed.[19] Eps1 is the only family member that is membrane-bound,[20] and Eug1 is the only yeast PDI with partial active site motifs (i.e., CXXS rather than CXXC).[19] Moreover, Mpd1, Mpd2, and Eps1, unlike Pdi1 and Eug1, only contain a single catalytic motif.[19] These differences, combined with variable expression levels during homeostasis and ER stress,[21] imply that there may be specific, yet uncovered, roles for each PDI in proteostasis.

Previously, we used divinyl sulfone (DVSF) – a cell-permeable, thiol-reactive cross-linker – to irreversibly trap the yeast thioredoxin Trx2 in complexes with several of its major redox partners, including the peroxiredoxin Tsa1, the thioredoxin reductase Trr1, and the

sulfiredoxin Srx1.[22–24] We reasoned that this approach could be useful in identifying potential redox partners and client proteins of the yeast PDIs, since they are members of the thioredoxin superfamily and many are targeted by thiol-reactive electrophiles.[25–30] Moreover, PDI client protein specificities and potential redox partners remain largely undefined. Here, we report that the PDIs from yeast are targets of DVSF and other bifunctional electrophiles. Using a DVSF-based cross-linking approach, we found that PDIs interact with known redox partners and putative client proteins in the secretory pathway as well as numerous factors involved in protein folding and quality control within the ER. Targeting of multiple ER proteostasis factors may account for impaired ER processes in DVSF-treated cells.

Experimental Procedures.

Expression Clones of Yeast PDIs, Putative PDI Interaction Partners, and Proteasome/ERAD Substrates.

Genes encoding epitope-tagged forms of PDIs and interaction partners were amplified from *S. cerevisiae* genomic DNA using Q5 PCR master mix (New England Biolabs) and the primers indicated in Supplementary Table 1 of the Supporting Information. PCR products were purified, digested with the appropriate restriction enzymes, and ligated into the yeast expression plasmids p415-GPD or p416-GPD.[31] Following transformation of the ligation products into the NEB5α *E. coli* cells (New England Biolabs), plasmids were isolated from cultures using a commercial miniprep procedure (Qiagen). Correct cloning was confirmed using restriction analysis and DNA sequencing. Constructs encoding HA-tagged CPY* and Deg1-β-galactosidase were kindly provided by Jeffrey Brodsky (University of Pittsburgh) and Jeffrey Laney (University of Massachusetts).[13, 32]

S. cerevisiae Cell Culture and Treatment.

BY4741 (wild-type) or *pdr5Δ* (*pdr5Δ::kan*^{r}) yeast strains were obtained from Open Biosystems. Yeast transformed with various expression or reporter plasmids were selected on the appropriate synthetic complete dropout medium (Sunrise Biosciences) at 30°C. The Hsp104-GFP strain was obtained from the GFP fusion library (Invitrogen) and cultured in YPD at 30°C. A strain containing a genomically integrated Ire1–3X-FLAG was kindly provided by Peter Walter (UCSF);[33] this strain was maintained in YPD at room temperature. All small molecules were dissolved in DMSO, and cells were treated with small molecules in mid-log phase. Total DMSO concentrations were ≤ 0.2% (v/v).

Preparation of Cellular Protein Lysates and Immunoblotting.

Cells treated with DVSF or other small molecules were lysed in TEGN buffer (20 mM Tris (pH 8.0), 0.5 mM EDTA, 10% glycerol, 50 mM NaCl, and protease inhibitor cocktail (G Biosciences)) using glass beads as previously described.[34] Protein concentrations in lysates were quantified using Bradford reagent (Biorad) to ensure equal loading of samples. Proteins (5–20 μg) were incubated with reducing SDS-PAGE loading dyes for 5 min at 95°C prior to resolution by SDS-PAGE. Proteins were electrophoretically transferred to PVDF membranes at 4°C. Subsequently, membranes were blocked with TBS-T buffer (100 mM Tris (pH 7.5), 150 mM NaCl, and 0.1% (v/v) Tween 20)

containing 5% non-fat dry milk and probed with primary antibodies against the FLAG tag (Sigma, M2 mouse monoclonal), the HA tag (Roche, rat monoclonal), Pgk1 (Invitrogen, mouse monoclonal), or β-galactosidase (Developmental Studies Hybridoma Bank, mouse monoclonal) overnight at 4°C. Membranes were washed with TBS-T three times for 10 min, incubated with appropriate secondary antibodies linked to horseradish peroxidase (Cell Signaling) for 45 min at room temperature, and washed four times with TBS-T for 15 min. Chemiluminescence signal was detected on a Chemidoc Touch Imaging System (Biorad).

Immunoprecipitations of PDIs with Interactions Partners.

For protein identification and co-association experiments, protein lysates (0.1–2 mg) from cells expressing FLAG-tagged Pdi1 or Eug1 that had been treated with DMSO or DVSF were incubated on a rotary mixer with EZView anti-FLAG beads (Sigma, M2) for 4 h at 4°C. Beads were pelleted for 1 min at 10000 × g and, after supernatant removal, were washed six times with 500 μL TEGN containing 0.1% SDS and 0.5% sodium deoxycholate. After the last wash, beads were incubated in 20–30 μL of elution buffer (50 mM Tris (pH 7.5), 150 mM NaCl, 0.5 mg/mL 3X FLAG peptide (Sigma)) at room temperature for 30 min. After centrifuging the samples to pellet beads, the supernatant was recovered and incubated with reducing SDS-PAGE loading dyes for 5 min at 95°C. Proteins were subsequently resolved on SDS-PAGE and stained with Coomassie blue (to extract bands for protein identification) or transferred to PVDF for immunoblotting with antibodies against HA and FLAG tags (as described above).

Proteomic Analysis of Pdi1 and Eug1 Interaction Partners.

Identification of proteins associated with Pdi1 and Eug1 following immunoprecipitation was carried out on two biological replicates by MS Bioworks (Ann Arbor, MI). Briefly, proteins in four excised gel bands (ranging from approximately 125–300 kDa) were subjected to in-gel alkylation with iodoacetamide and digestion with trypsin. Resulting peptides were analyzed by nano LC-MS/MS with a Waters M-Class LC system interfaced to a ThermoFisher Fusion Lumos mass spectrometer. Peptides were loaded on a trapping column and eluted over a 75 μm analytical column at 350 nL/min; both columns contained Luna C18 resin (Phenomenex). The mass spectrometer was operated in data-dependent mode, with the Orbitrap set at 60,000 FWHM and 15,000 FWHM for MS and MS/MS, respectively. The instrument was run with a 3 s cycle for MS and MS/MS. Data were searched using Mascot (Matrix Science) for tryptic fragments of proteins from *Saccharomyces cerevisiae* using the UniProt database, accounting for the following fixed and variable modifications: carbamidomethylation of cysteine (fixed), oxidation of methionine (variable), *N*-terminal acetylation (variable), cyclization of *N*-terminal glutamate (variable), and deamidation of asparagine and glutamine (variable). Mascot files were analyzed in Scaffold (Proteome Software) to create non-redundant protein lists for each sample. Data were filtered using 1% protein and peptide false discovery rates. Cysteine-containing proteins that yielded three or more spectral counts in both biological replicates were further studied for subcellular localization using information available on the *Saccharomyces* genome database (http://www.yeastgenome.org) and associated databases to subtract out cytosolic, nuclear, and mitochondrial proteins. These refined lists of proteins were used to compare the overlap between Eug1- and Pdi1-associated proteins and were also

analyzed for biological process using the Gene Ontology Enrichment Analysis site (https://biit.cs.ut.ee/gprofiler/gost).[35] Proteomics data have been deposited to the MassIVE database (https://massive.ucsd.edu/) under numbers MSV000088257 (Pdi1-associated proteins, ftp://massive.ucsd.edu/MSV000088257/ and MSV000088258 (Eug1-associated proteins, ftp://massive.ucsd.edu/MSV000088258/).

Analysis of Hsp104-GFP Aggregation.

Yeast expressing a genomically encoded Hsp104-GFP fusion were grown to mid-log phase at 30°C and treated with DVSF or heat shocked at 42°C for 15 min. Cells were pelleted and washed with deionized water prior to imaging for GFP on an Olympus IX73 microscope at 100X under oil immersion.

Analysis of Stress-Responsive Gene Expression.

β-Galactosidase reporter constructs for monitoring the Hsf1-regulated heat shock response, the Yap1-regulated antioxidant response, and the Hac1-regulated unfolded protein response have been reported previously.[36, 37] Wild-type (BY4741) yeast cells transformed with these constructs were grown to mid-log phase in SC-Ura medium and treated with varying doses of DVSF for 1 h at 30°C. For some experiments, cells were pre-treated with DVSF for 30 min prior to treatment with tunicamycin (Tm) for 1 h. β-galactosidase activity was measured by pelleting 1.5 mL of cells, washing pellets once with 500 μL water, before pelleting cells again and resuspending in 750 μL buffer Z (100 mM Na_3PO_4 (pH 7), 10 mM KCl, 1 mM $MgSO_4$, and 50 mM β-mercaptoethanol). Subsequently, 75 μL of chloroform and 37.5 μL 0.1% (w/v) SDS were added, and samples were briefly vortexed to lyse. Lysates were incubated at 30°C for 5 min prior to incubation with 150 μL 4 mg/mL *o*-nitrophenyl-β-galactoside (dissolved in buffer Z) for 30–120 min at 30°C. Reactions were terminated by adding 375 μL 1 M Na_2CO_3 and centrifuged for 3 min at 21000 × g to clear debris. The absorbance of the supernatant was measured at 420 and 550 nm, along with the OD_{600} of the original culture at the time of assay. Miller units were calculated using the expression below, where T is the time of the reaction and V is the volume of cell culture used in the assay.

$$\text{Miller Units} = 1000 \times [A_{420} - (1.75 \times A_{550})]/[T \times V \times OD_{600}]$$

Miller units were normalized as fold change in relation to the control sample (which was set at 1). The effect of DVSF and Tm on *HAC1* splicing was monitored by reverse transcription polymerase chain reaction (RT-PCR) as previously described.[38]

Results.

Previously, we and others have reported that thioredoxin superfamily members, including thioredoxins and PDIs, are targets of the bifunctional electrophile DVSF.[22, 23, 39, 40] Since the redox partners and client proteins of particular PDIs remain largely undefined, we employed a cross-linking approach to identify potential redox partners and client proteins of the PDIs in *S. cerevisiae*. To this end, we over-expressed FLAG-tagged forms of each PDI encoded by yeast using a constitutive GPD promoter and tested whether each

was a target of DVSF (Fig. 1A). Four of the PDIs in yeast (Pdi1, Mpd1, Mpd2, and Eug1) underwent dose-dependent cross-linking (as indicated by shifts in molecular weight) following cell treatment with DVSF, with cross-linking of Pdi1 and Eug1 to other proteins being particularly pronounced (Fig. 1B). We were unable to observe strong over-expression of FLAG-tagged Eps1, the remaining PDI encoded in yeast (data not shown), perhaps due to it being a membrane protein;[20] thus, it was not included in further analysis. General cross-linking of proteins to PDIs was comparable under homeostatic growth conditions or when cells were pre-treated with two ER stressors, including the disulfide reductant β-mercaptoethanol and the protein glycosylation inhibitor tunicamycin (Tm, Supp. Fig. S1), similar to effects observed where disulfide bond levels do not change significantly upon ER stress in mammalian cells.[41] Each of these proteins also underwent protein cross-linking in cells treated with toxic concentrations of the thiol-reactive bifunctional electrophiles nitrogen mustard (mechlorethamine, HN2) and 1,2,3,4-diepoxybutane (DEB) but not with bifunctional electrophile diethylacetylene dicarboxylate (DAD, Supp. Fig. S2).

Given the extensive cross-linking observed when cells expressing FLAG-tagged Pdi1 and Eug1 were treated with DVSF and other cross-linkers, we sought to identify the proteins that were associated with these PDIs using DVSF as the cross-linker. FLAG-tagged Eug1 and Pdi1 were immunoprecipitated from lysates of DVSF-treated cells, and associated proteins were identified from bands excised from the gels (Fig. 2A). After eliminating proteins that gave fewer than three spectral counts in two biological replicates and those that did not contain cysteine, we further refined our data sets by removing proteins that are localized to the cytosol and other subcellular regions that are not part of the secretory pathway, focusing on proteins that reside within the ER, Golgi, and/or vacuole or at the cell periphery (Fig. 2B). Following this subtraction step, 79 proteins were identified as putative interaction partners of Pdi1, and 55 proteins were isolated in complexes with Eug1 (Fig. 2C, Table 1). While we originally anticipated to find different client proteins and interaction partners for Eug1 and Pdi1, we observed considerable overlap between the Pdi1 and Eug1 datasets, with Pdi1 showing a greater number of associated proteins (Fig. 2C, Table 1). The finding that Pdi1 has a broader number of interaction partners is in keeping with its role as the only essential PDI in yeast.[19] To further explore the functional classification of these PDI interaction partners, we compared gene ontology predictions for those proteins associated with Eug1 and Pdi1, revealing that both PDIs interact with proteins involved in similar processes (Fig. 2D). Of note, only two of the interaction partners that were unique to Pdi1–Gpi16 and Pma1–are essential. Moreover, there was no apparent enrichment for particular protein folds or post-translational modifications in the Pdi1 dataset (data not shown).

To confirm our proteomics findings, we conducted co-immunoprecipitation experiments by co-expressing HA-tagged Pdi1 in cells with FLAG-tagged interaction partners and subjecting these cells to DVSF treatment. As noted in Fig. 3 and Table 1, Pdi1 formed cross-linked complexes with a known substrate/client protein (Prc1) and another putative client protein (Exg1).[42] Moreover, Pdi1 formed a broad range of cross-linked complexes with itself, other PDIs (i.e., Eug1, Mpd1, and Mpd2), the ER disulfide oxidase and known redox partner Ero1, and other proteins involved in ER protein quality control (i.e., Cpr5 and Yos9).[42] These findings, combined with the protein identification results, indicate that Pdi1 interacts with other proteins involved in ER proteostasis and perhaps participates in redox

partnerships with other chaperones. Since the results revealed that multiple, ER-resident proteostasis factors are subject to covalent modification by thiol-reactive electrophiles, we examined whether DVSF alters ER quality control mechanisms and stress response pathways.

Of these quality control processes, ERAD plays a key role in targeting improperly folded proteins in the ER lumen to the cytosol for degradation by the proteasome. Of note, several cross-linked interaction partners of Pdi1 and Eug1 proteins, including Yos9 and Mnl1/Htm1, participate in ERAD.[43] We reasoned that it is likely these proteins are inactive upon DVSF treatment, thereby inhibiting ERAD. To this end, we monitored the turnover of a common ERAD substrate, a mutant form of Prc1 (or carboxypeptidase Y) called CPY*, upon treatment of cells with DVSF and the protein synthesis inhibitor cycloheximide (CHX).[44] Dose-dependent cross-linking of CPY* was observed, as would be anticipated since we identified Prc1 as a target of DVSF in our proteomics work. Moreover, at higher DVSF doses, an accumulation of both the CPY* monomer and CPY* cross-linked complexes occurred (Fig. 4A). Quantification of CPY* levels at the highest DVSF concentration were nearly identical with or without treatment with CHX, indicating that DVSF impairs turnover of CPY* by ERAD. This effect was likely not due to inhibition of the proteasome by DVSF, since DVSF decreased levels of degron-linked β-galactosidase, suggesting that it upregulates proteasome activity (Fig. 4B). In contrast, treatment of cells with the proteasome inhibitor MG132 led to an increase in the levels of the degron-β-galactosidase (Fig. 4B). Collectively, our results suggest that one (or more) steps in the ERAD pathway is (or are) perturbed by DVSF, presumably within the ER lumen.

Our proteomics results indicated multiple ER-resident molecular chaperones are targeted by DVSF, suggesting that proteostasis in this organelle may be affected. As a general readout of proteostasis that reports on cytosolic and ER protein misfolding and aggregation,[45, 46] we monitored accumulation of a GFP-tagged form of the disaggregase Hsp104 in puncta following treatment with DVSF or exposure to heat shock. Puncta were observed at 1 μM DVSF and became more numerous in cells at higher DVSF concentrations (Fig. 5A). Moreover, the puncta were much more intense when compared with those caused by heat shock (Fig. 5A). To determine how DVSF influences stress-responsive gene expression, we used β-galactosidase reporters that are regulated by the antioxidant response transcription factor Yap1 (ARE::lacZ), the heat shock transcription factor Hsf1 (HSE::lacZ), and the unfolded protein response (UPR)/ER stress response transcription factor Hac1 (UPRE::lacZ). DVSF treatment led to a dose-dependent increase in Yap1- and Hsf1-regulated reporter activity at levels comparable to positive controls (Fig. 5B, Supp. Fig. S3), but did not induce the Hac1-regulated UPR reporter, despite extensive damage to ER-resident proteostasis factors as determined through our earlier proteomics work. To further investigate this apparent lack of UPR induction, we tested whether DVSF inhibits the UPR. Pretreatment of cells with DVSF led to a dose-dependent decrease in Hac1 reporter activity triggered by tunicamycin (Tm, Fig. 5C), as well as a decrease in Tm-induced *HAC1* splicing (Fig. 5D). Short-term coadministration of DVSF and Tm did not result in cell death, suggesting that the lack of ER-stress response induction is not due to toxicity resulting from short-term administration of the two molecules (Supp. Fig. S4). Instead, it is likely that DVSF blocks the signaling pathway that activates the ER-stress response, as

the *HAC1*-splicing factor Ire1 undergoes dose-dependent cross-linking in cells treated with DVSF, indicating it is a DVSF target at the concentrations used (Fig. 5E).

Discussion.

Here, we report that baker's yeast PDIs are targets of thiol-reactive bifunctional electrophiles and use one of these molecules, DVSF, to identify putative redox partners of PDIs. Many cross-linked binding partners identified for Pdi1 and Eug1 play defined roles in the lysosome or at the cell periphery, while others function in protein quality control within the ER, prompting us to investigate effects on ER proteostasis. DVSF treatment blocked turnover of the ERAD substrate CPY* and conversely enhanced clearance of a cytosolic proteasome substrate. Moreover, we found that, despite targeting numerous chaperones in the ER, DVSF inhibited ER stress signaling, potentially by covalently modifying and inactivating Ire1. Collectively, we demonstrate that bifunctional electrophiles target many proteins within the ER proteostasis network, suggesting that the combined effects of their modification and presumed inactivation have far-reaching impacts on secretory pathway quality control.

The need to identify and characterize specific interaction partners and clients of PDIs has long been acknowledged, prompting the development of different experimental strategies ranging from yeast two hybrid screening to pulldown approaches.[10, 11, 40, 47–51] While many studies have focused on mechanistic investigation of one specific interaction partner of PDIs, those relying on biochemical approaches have provided more substantive lists of PDI interaction partners.[10, 11, 40, 48] When viewing the results of such global profiling studies in combination with our results, we gain a more comprehensive picture of the key interaction partners of PDIs. Our approach, while differing from others in the way we capture interactions between PDIs and their partners with a thiol-reactive cross-linker, yielded a broader data set that aligns with subsets of hits from earlier studies. In addition to PDIs interacting with putative client proteins that traffic to downstream locations in the secretory pathway, we found that PDIs also interact with themselves, other chaperones (e.g., the ER-resident Hsp70/BiP, several DnaJ proteins, and peptidyl-prolyl isomerases), and their upstream redox partner Ero1, a protein that is known to transfer disulfide bonds directly to PDIs.[42, 52] The majority of these interactions are short-lived, given that a cross-linking agent is critical for observing the interaction.

The observation that both Eug1 and Pdi1 become cross-linked to other PDIs from yeast closely matches a recent study by Cobb et al., where one PDI became cross-linked to two others in *Plasmodium falciparum* treated with DVSF.[40] Such formation of mixed PDI complexes suggests these enzymes are doing one of two tasks during electrophilic stress. First, PDIs may form mixed dimers with one another when folding proteins, potentially participating in intersubunit redox exchange between individual PDI protomers. Short-lived dimerization of individual PDI family members has been observed,[53] as has enhanced activity when certain PDIs are added in combination.[54] Both Pdi1 and Eug1 formed cross-linked complexes with other PDIs, suggesting that these proteins form heterogeneous complexes with one another and perhaps carry out intersubunit electron transfers. As an alternate explanation, individual PDIs may constantly be surveilling the redox status of cysteines in many proteins within the ER lumen, even that of other PDI

family members. PDIs are thought to monitor both native and non-native disulfides in protein substrates, lending some support for this model.[55] With this explanation, PDIs could become cross-linked with one another merely by recognizing the abnormal state caused by DVSF-mediated alkylation of another protein, ultimately leading to cross-linked complex formation. The same explanations may account for why a broad subset of molecular chaperones and proteostasis factors associate with Pdi1 and Eug1 upon the addition of DVSF.[11, 40, 48] Trying to ascertain which of these two models – redox collaboration versus damage recognition – accounts for cross-linked complex formation in cells represents a technical challenge that may be difficult to address. Regardless, the extensive damage to the ER proteostasis network caused by DVSF and related electrophiles implies that they may have considerable impacts on quality control within the organelle and those locations downstream in the secretory pathway.

Much like client protein pools for specific PDIs, the impact of electrophiles and other thiol-reactive molecules on ER quality control mechanisms and stress signaling remains poorly understood. It is well-established that molecular chaperones, especially the ER-resident Hsp70 Kar2/BiP and PDIs, play key roles in ER stress response regulation and attenuation.[56, 57] Moreover, the work presented herein reveals many ER-resident chaperones are subject to electrophilic modification. Organic electrophiles and other thiol-reactive molecules often target key thiols within these proteins to inhibit their activity.[25–30] Because PDIs and their cross-linked partners regulate various aspects of secretory pathway protein quality control, we expected that DVSF would activate, rather than inhibit, the Hac1-mediated ER stress response. In some instances, the Ire1 arm of the ER stress pathway may be activated by electrophiles, like the lipid peroxidation product 4-hydroxynonenal;[58, 59] however, our results suggest that response activation by electrophiles is potentially tempered by Ire1 modification and inactivation. Along these lines, Ire1 can be modified and inactivated by *S*-nitrosating agents and other Michael acceptors.[60, 61] Given its low abundance relative to PDIs and other ER chaperones, targeting Ire1 by electrophilic modification represents a potential way for pharmacologically perturbing the UPR.

As with ER stress response signaling, our findings suggest that DVSF inactivates parts of the ERAD pathway, while simultaneously activating degradation of degron-linked reporter enzyme in the cytosol. For many misfolded proteins in the ER, ERAD depends on the function of two proteins that we found associated in high molecular complexes with Pdi1 – the mannosidase Mnl1 (also known as Htm1) and the lectin Yos9 – both of which contribute to targeting ERAD substrates for retrotranslocation followed by polyubiquitylation and proteasomal degradation in the cytosol. Pdi1 forms a complex with Mnl1, where it allows for glycan trimming in advance of ERAD.[12, 14–16] By contrast, Yos9 recognizes the trimmed oligosaccharide generated by Mnl1 and interacts with the Hrd1 complex, leading to retrotranslocation and ubiquitylation of certain ERAD substrates.[62–64] Coincidentally, the Yos9 structure contains four disulfide bonds, indicating that it may interact with PDIs as a client protein.[64] The interaction of Pdi1 with two critical ERAD factors in DVSF-treated cells potentially contributes to their impaired folding and/or covalent inactivation, thereby offering an explanation for why degradation of the ERAD substrate CPY* is impaired at high DVSF concentrations. Moreover, the observed ERAD defects may be exacerbated by UPR inhibition, since these two processes are interrelated in some cases.[21] At the

same DVSF concentrations that impair ERAD, general proteasome activity appears to be upregulated, although we cannot rule out that DVSF decreases expression of the degron-linked β-galactosidase reporter at the transcriptional level with the experiments performed. A mechanism for this likely upregulation of proteasome activity (for instance, via Rpn4 activation) by DVSF remains unresolved.[65]

Our results suggest cell-permeable protein cross-linkers impact proteostasis in multiple cellular compartments. In the ER, we found that many proteins that comprise the protein folding and quality control machinery were susceptible to electrophilic modification by DVSF. Given that PDIs are covalently modified by targeted electrophiles of potential therapeutic benefit,[26–30] we suggest that other secretory pathway proteostasis factors may also be druggable in this manner. Since PDIs and other ER-resident proteins involved in protein quality control have far-reaching roles in homeostasis and disease, developing a suite of thiol-targeted probes specific for individual ER proteostasis network members may be useful in further understanding the biological roles of these proteins and may aid in drug development efforts for diseases with imbalanced secretory pathway function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements.

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Abbreviations.

PDI	protein disulfide isomerase
DVSF	divinyl sulfone
RT-PCR	reverse transcription-polymerase chain reaction
ERAD	ER-associated protein degradation
CPY*	mutated form of carboxypeptidase Y
CHX	cycloheximide
GFP,	green fluorescent protein

UPR,	unfolded protein response
Tm	tunicamycin

References.

1. Hartl FU; Bracher A; Hayer-Hartl M, Molecular chaperones in protein folding and proteostasis. Nature 2011, 475 (7356), 324–32. [PubMed: 21776078]
2. Balch WE; Morimoto RI; Dillin A; Kelly JW, Adapting proteostasis for disease intervention. Science 2008, 319 (5865), 916–9. [PubMed: 18276881]
3. Brodsky JL; Skach WR, Protein folding and quality control in the endoplasmic reticulum: Recent lessons from yeast and mammalian cell systems. Curr Opin Cell Biol 2011, 23 (4), 464–75. [PubMed: 21664808]
4. Feige MJ; Hendershot LM, Disulfide bonds in ER protein folding and homeostasis. Curr Opin Cell Biol 2011, 23 (2), 167–75. [PubMed: 21144725]
5. Lu J; Holmgren A, The thioredoxin superfamily in oxidative protein folding. Antioxid Redox Signal 2014, 21 (3), 457–70. [PubMed: 24483600]
6. Robinson PJ; Bulleid NJ, Mechanisms of Disulfide Bond Formation in Nascent Polypeptides Entering the Secretory Pathway. Cells 2020, 9 (9).
7. Matsusaki M; Kanemura S; Kinoshita M; Lee YH; Inaba K; Okumura M, The Protein Disulfide Isomerase Family: from proteostasis to pathogenesis. Biochim Biophys Acta Gen Subj 2020, 1864 (2), 129338. [PubMed: 30986509]
8. Schonbrunner ER; Schmid FX, Peptidyl-prolyl cis-trans isomerase improves the efficiency of protein disulfide isomerase as a catalyst of protein folding. Proc Natl Acad Sci U S A 1992, 89 (10), 4510–3. [PubMed: 1584784]
9. Mayer M; Kies U; Kammermeier R; Buchner J, BiP and PDI cooperate in the oxidative folding of antibodies in vitro. J Biol Chem 2000, 275 (38), 29421–5. [PubMed: 10893409]
10. Jessop CE; Watkins RH; Simmons JJ; Tasab M; Bulleid NJ, Protein disulphideisomerase family members show distinct substrate specificity: P5 is targeted to BiP client proteins. J Cell Sci 2009, 122 (Pt 23), 4287–95. [PubMed: 19887585]
11. Jansen G; Maattanen P; Denisov AY; Scarffe L; Schade B; Balghi H; Dejgaard K; Chen LY; Muller WJ; Gehring K; Thomas DY, An interaction map of endoplasmic reticulum chaperones and foldases. Mol Cell Proteomics 2012, 11 (9), 710–23. [PubMed: 22665516]
12. Sakoh-Nakatogawa M; Nishikawa S; Endo T, Roles of protein-disulfide isomerase-mediated disulfide bond formation of yeast Mnl1p in endoplasmic reticulum-associated degradation. J Biol Chem 2009, 284 (18), 11815–25. [PubMed: 19279007]
13. Grubb S; Guo L; Fisher EA; Brodsky JL, Protein disulfide isomerases contribute differentially to the endoplasmic reticulum-associated degradation of apolipoprotein B and other substrates. Mol Biol Cell 2012, 23 (4), 520–32. [PubMed: 22190736]
14. Gauss R; Kanehara K; Carvalho P; Ng DT; Aebi M, A complex of Pdi1p and the mannosidase Htm1p initiates clearance of unfolded glycoproteins from the endoplasmic reticulum. Mol Cell 2011, 42 (6), 782–93. [PubMed: 21700223]
15. Liu YC; Fujimori DG; Weissman JS, Htm1p-Pdi1p is a folding-sensitive mannosidase that marks N-glycoproteins for ER-associated protein degradation. Proc Natl Acad Sci U S A 2016, 113 (28), E4015–24. [PubMed: 27357682]
16. Pfeiffer A; Stephanowitz H; Krause E; Volkwein C; Hirsch C; Jarosch E; Sommer T, A Complex of Htm1 and the Oxidoreductase Pdi1 Accelerates Degradation of Misfolded Glycoproteins. J Biol Chem 2016, 291 (23), 12195–207. [PubMed: 27053108]
17. Eletto D; Eletto D; Dersh D; Gidalevitz T; Argon Y, Protein disulfide isomerase A6 controls the decay of IRE1alpha signaling via disulfide-dependent association. Mol Cell 2014, 53 (4), 562–576. [PubMed: 24508390]
18. Kozlov G; Maattanen P; Thomas DY; Gehring K, A structural overview of the PDI family of proteins. FEBS J 2010, 277 (19), 3924–36. [PubMed: 20796029]

19. Norgaard P; Westphal V; Tachibana C; Alsoe L; Holst B; Winther JR, Functional differences in yeast protein disulfide isomerases. J Cell Biol 2001, 152 (3), 553–62. [PubMed: 11157982]
20. Wang Q; Chang A, Eps1, a novel PDI-related protein involved in ER quality control in yeast. EMBO J 1999, 18 (21), 5972–82. [PubMed: 10545109]
21. Travers KJ; Patil CK; Wodicka L; Lockhart DJ; Weissman JS; Walter P, Functional and genomic analyses reveal an essential coordination between the unfolded protein response and ER-associated degradation. Cell 2000, 101 (3), 249–58. [PubMed: 10847680]
22. Naticchia MR; Brown HA; Garcia FJ; Lamade AM; Justice SL; Herrin RP; Morano KA; West JD, Bifunctional electrophiles cross-link thioredoxins with redox relay partners in cells. Chem Res Toxicol 2013, 26 (3), 490–7. [PubMed: 23414292]
23. Allan KM; Loberg MA; Chepngeno J; Hurtig JE; Tripathi S; Kang MG; Allotey JK; Widdershins AH; Pilat JM; Sizek HJ; Murphy WJ; Naticchia MR; David JB; Morano KA; West JD, Trapping redox partnerships in oxidant-sensitive proteins with a small, thiol-reactive cross-linker. Free Radic Biol Med 2016, 101, 356–366. [PubMed: 27816612]
24. Loberg MA; Hurtig JE; Graff AH; Allan KM; Buchan JA; Spencer MK; Kelly JE; Clodfelter JE; Morano KA; Lowther WT; West JD, Aromatic Residues at the Dimer-Dimer Interface in the Peroxiredoxin Tsa1 Facilitate Decamer Formation and Biological Function. Chem Res Toxicol 2019, 32 (3), 474–483. [PubMed: 30701970]
25. Carbone DL; Doorn JA; Kiebler Z; Petersen DR, Cysteine modification by lipid peroxidation products inhibits protein disulfide isomerase. Chem Res Toxicol 2005, 18 (8), 1324–31. [PubMed: 16097806]
26. Xu S; Butkevich AN; Yamada R; Zhou Y; Debnath B; Duncan R; Zandi E; Petasis NA; Neamati N, Discovery of an orally active small-molecule irreversible inhibitor of protein disulfide isomerase for ovarian cancer treatment. Proc Natl Acad Sci U S A 2012, 109 (40), 16348–53. [PubMed: 22988091]
27. Banerjee R; Pace NJ; Brown DR; Weerapana E, 1,3,5-Triazine as a modular scaffold for covalent inhibitors with streamlined target identification. J Am Chem Soc 2013, 135 (7), 2497–500. [PubMed: 23379904]
28. Cole KS; Grandjean JMD; Chen K; Witt CH; O'Day J; Shoulders MD; Wiseman RL; Weerapana E, Characterization of an A-Site Selective Protein Disulfide Isomerase A1 Inhibitor. Biochemistry 2018, 57 (13), 2035–2043. [PubMed: 29521097]
29. Allimuthu D; Adams DJ, 2-Chloropropionamide As a Low-Reactivity Electrophile for Irreversible Small-Molecule Probe Identification. ACS Chem Biol 2017, 12 (8), 2124–2131. [PubMed: 28613814]
30. Shergalis A; Xue D; Gharbia FZ; Driks H; Shrestha B; Tanweer A; Cromer K; Ljungman M; Neamati N, Characterization of Aminobenzylphenols as Protein Disulfide Isomerase Inhibitors in Glioblastoma Cell Lines. J Med Chem 2020, 63 (18), 10263–10286. [PubMed: 32830969]
31. Mumberg D; Muller R; Funk M, Yeast vectors for the controlled expression of heterologous proteins in different genetic backgrounds. Gene 1995, 156 (1), 119–22. [PubMed: 7737504]
32. Laney JD; Mobley EF; Hochstrasser M, The short-lived Matalpha2 transcriptional repressor is protected from degradation in vivo by interactions with its corepressors Tup1 and Ssn6. Mol Cell Biol 2006, 26 (1), 371–80. [PubMed: 16354707]
33. Rubio C; Pincus D; Korennykh A; Schuck S; El-Samad H; Walter P, Homeostatic adaptation to endoplasmic reticulum stress depends on Ire1 kinase activity. J Cell Biol 2011, 193 (1), 171–84. [PubMed: 21444684]
34. West JD; Stamm CE; Brown HA; Justice SL; Morano KA, Enhanced toxicity of the protein cross-linkers divinyl sulfone and diethyl acetylenedicarboxylate in comparison to related monofunctional electrophiles. Chem Res Toxicol 2011, 24 (9), 1457–9. [PubMed: 21812477]
35. Raudvere U; Kolberg L; Kuzmin I; Arak T; Adler P; Peterson H; Vilo J, g:Profiler: a web server for functional enrichment analysis and conversions of gene lists (2019 update). Nucleic Acids Res 2019, 47 (W1), W191–W198. [PubMed: 31066453]
36. Trott A; West JD; Klaic L; Westerheide SD; Silverman RB; Morimoto RI; Morano KA, Activation of heat shock and antioxidant responses by the natural product celastrol: transcriptional signatures of a thiol-targeted molecule. Mol Biol Cell 2008, 19 (3), 1104–12. [PubMed: 18199679]

37. Kabani M; Kelley SS; Morrow MW; Montgomery DL; Sivendran R; Rose MD; Gierasch LM; Brodsky JL, Dependence of endoplasmic reticulum-associated degradation on the peptide binding domain and concentration of BiP. Mol Biol Cell 2003, 14 (8), 3437–48. [PubMed: 12925775]
38. Bruch A; Klassen R; Schaffrath R, Unfolded Protein Response Suppression in Yeast by Loss of tRNA Modifications. Genes (Basel) 2018, 9 (11).
39. Araki K; Ushioda R; Kusano H; Tanaka R; Hatta T; Fukui K; Nagata K; Natsume T, A crosslinker-based identification of redox relay targets. Anal Biochem 2017, 520, 22–26. [PubMed: 28048978]
40. Cobb DW; Kudyba HM; Villegas A; Hoopmann MR; Baptista RP; Bruton B; Krakowiak M; Moritz RL; Muralidharan V, A redox-active crosslinker reveals an essential and inhibitable oxidative folding network in the endoplasmic reticulum of malaria parasites. PLoS Pathog 2021, 17 (2), e1009293. [PubMed: 33534803]
41. Bechtel TJ; Li C; Kisty EA; Maurais AJ; Weerapana E, Profiling Cysteine Reactivity and Oxidation in the Endoplasmic Reticulum. *ACS* Chem Biol 2020, 15 (2), 543–553.
42. Frand AR; Kaiser CA, Ero1p oxidizes protein disulfide isomerase in a pathway for disulfide bond formation in the endoplasmic reticulum. Mol Cell 1999, 4 (4), 469–77. [PubMed: 10549279]
43. Ninagawa S; George G; Mori K, Mechanisms of productive folding and endoplasmic reticulum-associated degradation of glycoproteins and non-glycoproteins. Biochim Biophys Acta Gen Subj 2021, 1865 (3), 129812. [PubMed: 33316349]
44. Finger A; Knop M; Wolf DH, Analysis of two mutated vacuolar proteins reveals a degradation pathway in the endoplasmic reticulum or a related compartment of yeast. Eur J Biochem 1993, 218 (2), 565–74. [PubMed: 8269947]
45. Preston GM; Guerriero CJ; Metzger MB; Michaelis S; Brodsky JL, Substrate Insolubility Dictates Hsp104-Dependent Endoplasmic-Reticulum-Associated Degradation. Mol Cell 2018, 70 (2), 242–253 e6. [PubMed: 29677492]
46. Doonan LM; Guerriero CJ; Preston GM; Buck TM; Khazanov N; Fisher EA; Senderowitz H; Brodsky JL, Hsp104 facilitates the endoplasmic-reticulum-associated degradation of disease-associated and aggregation-prone substrates. Protein Sci 2019, 28 (7), 1290–1306. [PubMed: 31050848]
47. Ko HS; Uehara T; Nomura Y, Role of ubiquilin associated with protein-disulfide isomerase in the endoplasmic reticulum in stress-induced apoptotic cell death. J Biol Chem 2002, 277 (38), 35386–92. [PubMed: 12095988]
48. Meunier L; Usherwood YK; Chung KT; Hendershot LM, A subset of chaperones and folding enzymes form multiprotein complexes in endoplasmic reticulum to bind nascent proteins. Mol Biol Cell 2002, 13 (12), 4456–69. [PubMed: 12475965]
49. Kadokura H; Saito M; Tsuru A; Hosoda A; Iwawaki T; Inaba K; Kohno K, Identification of the redox partners of ERdj5/JPDI, a PDI family member, from an animal tissue. Biochem Biophys Res Commun 2013, 440 (2), 245–50. [PubMed: 24055038]
50. Stopa JD; Baker KM; Grover SP; Flaumenhaft R; Furie B, Kinetic-based trapping by intervening sequence variants of the active sites of protein-disulfide isomerase identifies platelet protein substrates. J Biol Chem 2017, 292 (22), 9063–9074. [PubMed: 28364042]
51. Eriksson O; Chiu J; Hogg PJ; Atkinson JP; Liszewski MK; Flaumenhaft R; Furie B, Thiol isomerase ERp57 targets and modulates the lectin pathway of complement activation. J Biol Chem 2019, 294 (13), 4878–4888. [PubMed: 30670593]
52. Sevier CS; Cuozzo JW; Vala A; Aslund F; Kaiser CA, A flavoprotein oxidase defines a new endoplasmic reticulum pathway for biosynthetic disulphide bond formation. Nat Cell Biol 2001, 3 (10), 874–82. [PubMed: 11584268]
53. Okumura M; Noi K; Kanemura S; Kinoshita M; Saio T; Inoue Y; Hikima T; Akiyama S; Ogura T; Inaba K, Dynamic assembly of protein disulfide isomerase in catalysis of oxidative folding. Nat Chem Biol 2019, 15 (5), 499–509. [PubMed: 30992562]
54. Kimura T; Hosoda Y; Sato Y; Kitamura Y; Ikeda T; Horibe T; Kikuchi M, Interactions among yeast protein-disulfide isomerase proteins and endoplasmic reticulum chaperone proteins influence their activities. J Biol Chem 2005, 280 (36), 31438–41. [PubMed: 16002399]
55. Zheng J; Gilbert HF, Discrimination between native and non-native disulfides by protein-disulfide isomerase. J Biol Chem 2001, 276 (19), 15747–52. [PubMed: 11278905]

56. Karagoz GE; Acosta-Alvear D; Walter P, The Unfolded Protein Response: Detecting and Responding to Fluctuations in the Protein-Folding Capacity of the Endoplasmic Reticulum. Cold Spring Harb Perspect Biol 2019, 11 (9).
57. Eletto D; Chevet E; Argon Y; Appenzeller-Herzog C, Redox controls UPR to control redox. J Cell Sci 2014, 127 (Pt 17), 3649–58. [PubMed: 25107370]
58. West JD; Marnett LJ, Alterations in gene expression induced by the lipid peroxidation product, 4-hydroxy-2-nonenal. Chem Res Toxicol 2005, 18 (11), 1642–53. [PubMed: 16300372]
59. Vladykovskaya E; Sithu SD; Haberzettl P; Wickramasinghe NS; Merchant ML; Hill BG; McCracken J; Agarwal A; Dougherty S; Gordon SA; Schuschke DA; Barski OA; O'Toole T; D'Souza SE; Bhatnagar A; Srivastava S, Lipid peroxidation product 4-hydroxy-trans-2-nonenal causes endothelial activation by inducing endoplasmic reticulum stress. J Biol Chem 2012, 287 (14), 11398–409. [PubMed: 22228760]
60. Nakato R; Ohkubo Y; Konishi A; Shibata M; Kaneko Y; Iwawaki T; Nakamura T; Lipton SA; Uehara T, Regulation of the unfolded protein response via S-nitrosylation of sensors of endoplasmic reticulum stress. Sci Rep 2015, 5, 14812. [PubMed: 26446798]
61. Waller DD; Jansen G; Golizeh M; Martel-Lorion C; Dejgaard K; Shiao TC; Mancuso J; Tsantrizos YS; Roy R; Sebag M; Sleno L; Thomas DY, A Covalent Cysteine-Targeting Kinase Inhibitor of Ire1 Permits Allosteric Control of Endoribonuclease Activity. Chembiochem 2016, 17 (9), 843–51. [PubMed: 26792008]
62. Gauss R; Jarosch E; Sommer T; Hirsch C, A complex of Yos9p and the HRD ligase integrates endoplasmic reticulum quality control into the degradation machinery. Nat Cell Biol 2006, 8 (8), 849–54. [PubMed: 16845381]
63. Clerc S; Hirsch C; Oggier DM; Deprez P; Jakob C; Sommer T; Aebi M, Htm1 protein generates the N-glycan signal for glycoprotein degradation in the endoplasmic reticulum. J Cell Biol 2009, 184 (1), 159–72. [PubMed: 19124653]
64. Wu X; Siggel M; Ovchinnikov S; Mi W; Svetlov V; Nudler E; Liao M; Hummer G; Rapoport TA, Structural basis of ER-associated protein degradation mediated by the Hrd1 ubiquitin ligase complex. Science 2020, 368 (6489).
65. Hanna J; Finley D, A proteasome for all occasions. FEBS Lett 2007, 581 (15), 2854–61. [PubMed: 17418826]

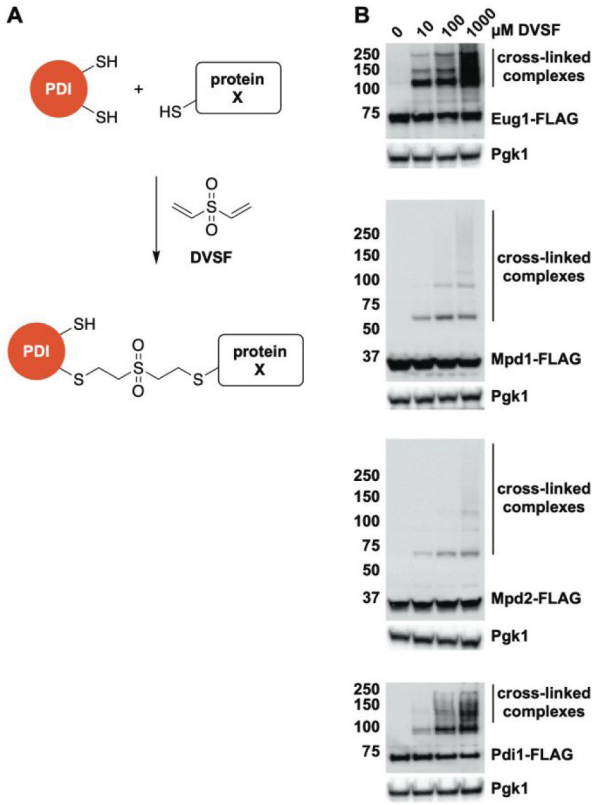

Figure 1. PDIs Form Cross-Linked Complexes with Other Proteins in Cells Treated With DVSF.

(A) Scheme of DVSF-mediated cross-linking of thiols between PDIs and interaction partners. (B) Wild-type yeast cells (BY4741) expressing FLAG-tagged PDIs were treated with indicated doses of DVSF for 1 h at 30°C. Protein lysates were resolved on SDS-PAGE and detected by immunoblot with an antibody against the FLAG tag to monitor accumulation of high molecular weight (i.e., cross-linked) complexes. Pgk1 levels were monitored as a loading control. Results are representative of three independent experiments.

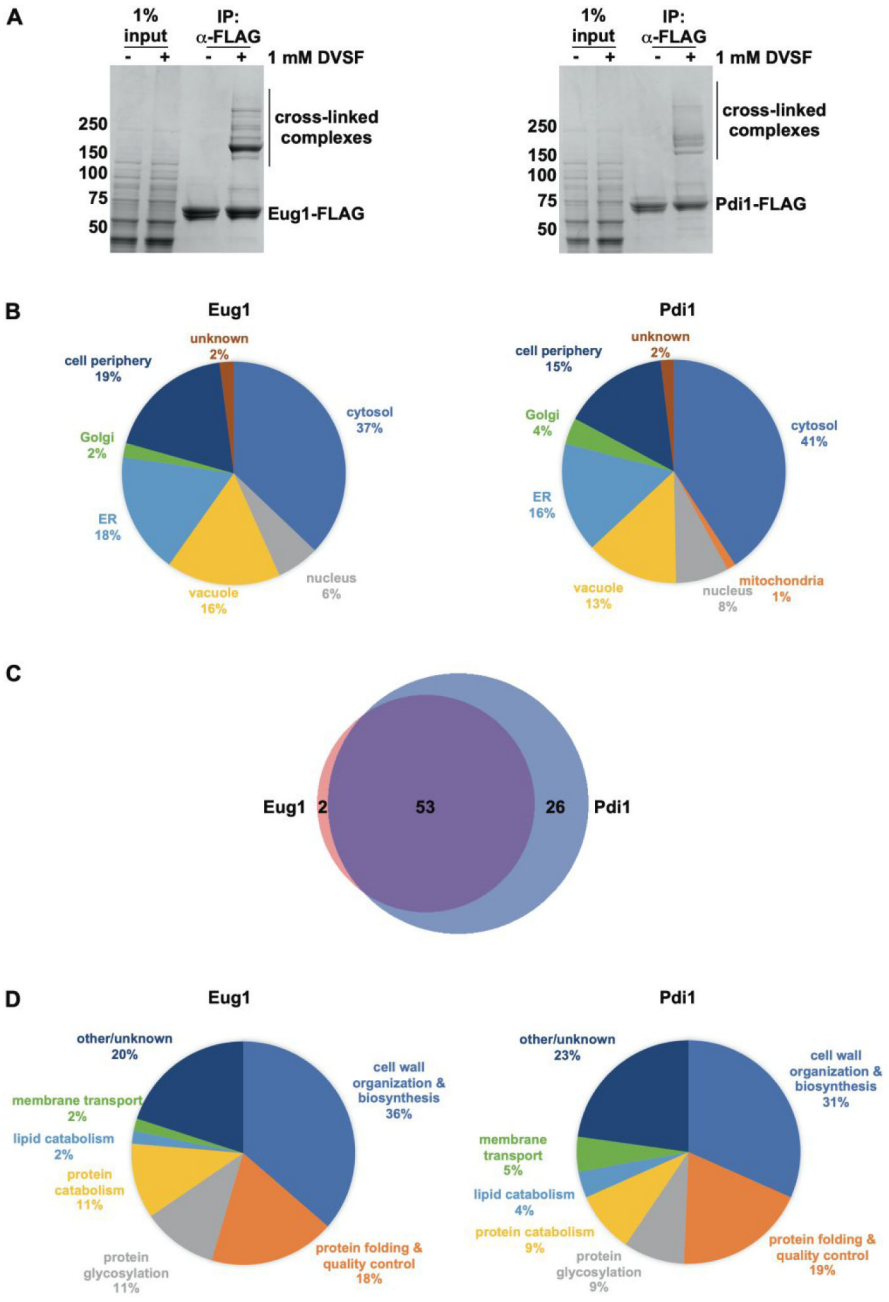

Figure 2. Pdi1 and Eug1 Are Cross-Linked to Many of the Same Interaction Partners.

(A) Isolation of cross-linked complexes for identification of Eug1- and Pdi1-associated proteins. FLAG-tagged Pdi1 or Eug1 was immunoprecipitated from 2 mg of lysates from cells treated with vehicle or 1 mM DVSF for 1 h. Proteins were resolved by SDS-PAGE and visualized with Coomassie blue. Bands from cross-linked complexes were excised for protein identification. Proteins identified and further characterized were the same in two independent biological replicates. (B) Subcellular localization of hits from protein identification. Proteins that were cytosolic, nuclear, or mitochondrial according to information in the *Saccharomyces* Genome Database (https://www.yeastgenome.org) were excluded from further analysis. (C) Overlap among Eug1- and Pdi1-associated proteins.

For a list of protein names, refer to Table 1. (D) Molecular processes that Eug1- and Pdi1-associated proteins participate in. For a list of protein names, refer to Table 1.

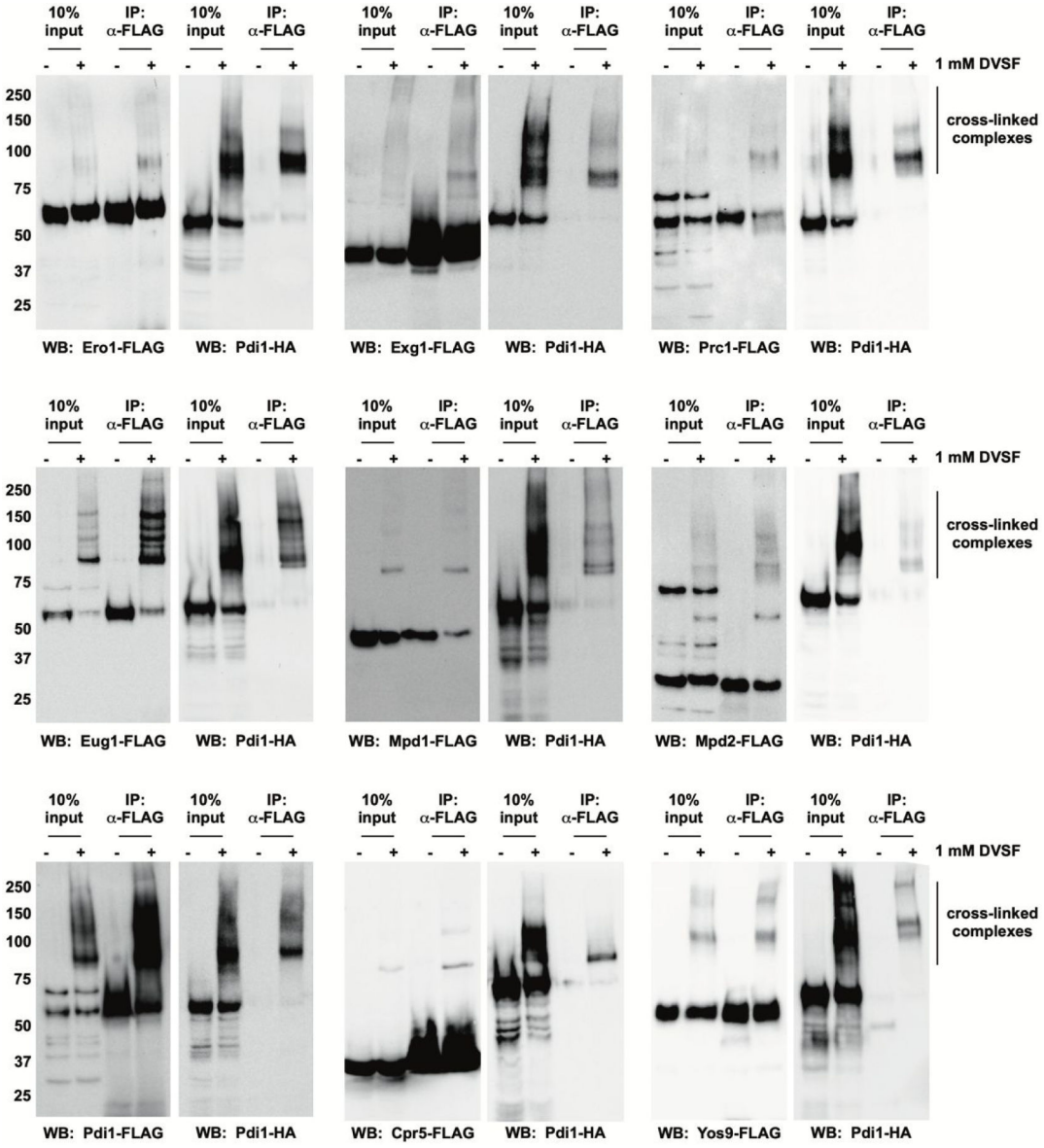

Figure 3. Co-Immunoprecipitation of Pdi1 with Putative Client Proteins and ER-Resident Chaperones in DVSF-Treated Cells.

Verification of proteomics results was conducted with a selected number of Pdi1-associated proteins. Wild-type cells (BY4741) expressing HA-tagged Pdi1 and FLAG-tagged binding partners were treated with 1 mM DVSF for 1 h at 30°C. Complexes were immunoprecipitated from lysates using anti-FLAG beads, and immunoblots against the HA and FLAG tags were used to detect cross-linked complexes between Pdi1 and its interaction partners. Results are representative of at least two independent experiments.

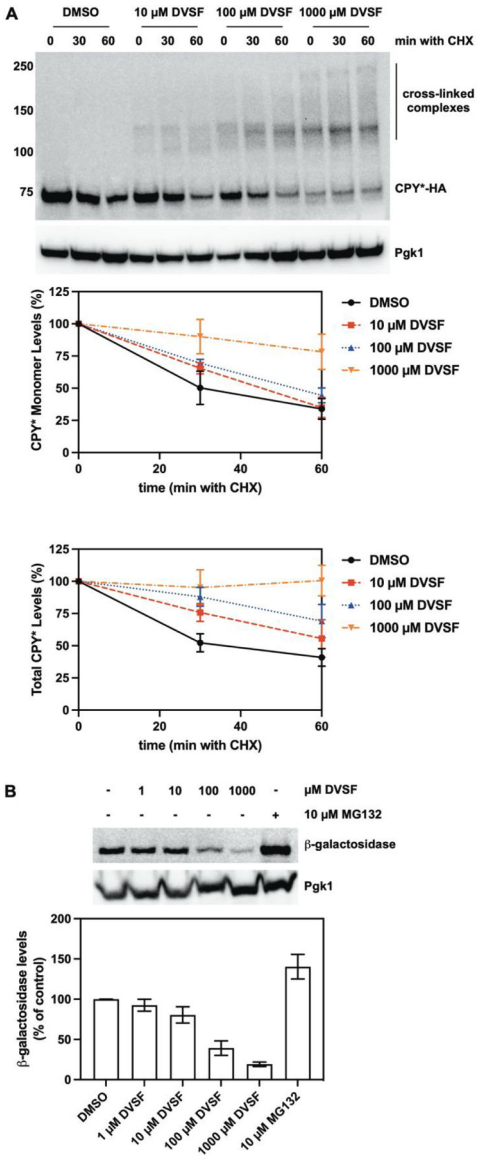

Figure 4. DVSF Decreases Degradation of the ERAD Substrate CPY* but Enhances Degradation of a Degron-Linked β-Galactosidase.

(A) Yeast cells (BY4741) expressing HA-tagged CPY* were treated for 1 h with the indicated concentrations of DVSF prior to a chase with 200 μg/mL cycloheximide (CHX) for the indicated times. Protein lysates were resolved by SDS-PAGE, and CPY* was detected by immunoblotting with an antibody against the HA epitope. Pgk1 levels were monitored as a loading control. (B) Yeast cells lacking the efflux pump Pdr5 (*pdr5Δ*, to enable accumulation of the proteasome inhibitor MG132) and expressing a degron-linked β-galactosidase were treated with the indicated concentrations of DVSF or MG132 for 1 h. Protein lysates were resolved by SDS-PAGE, and β-galactosidase levels were detected by immunoblotting. Pgk1 levels were monitored as a loading control. Results are representative of four independent experiments. Band intensities for monomeric CPY*, total CPY* (which includes its cross-linked complexes), or β-galactosidase were quantified using Fiji (https://

imagej.net/software/fiji/). Graphs show the average of the four trials ± standard error of the mean.

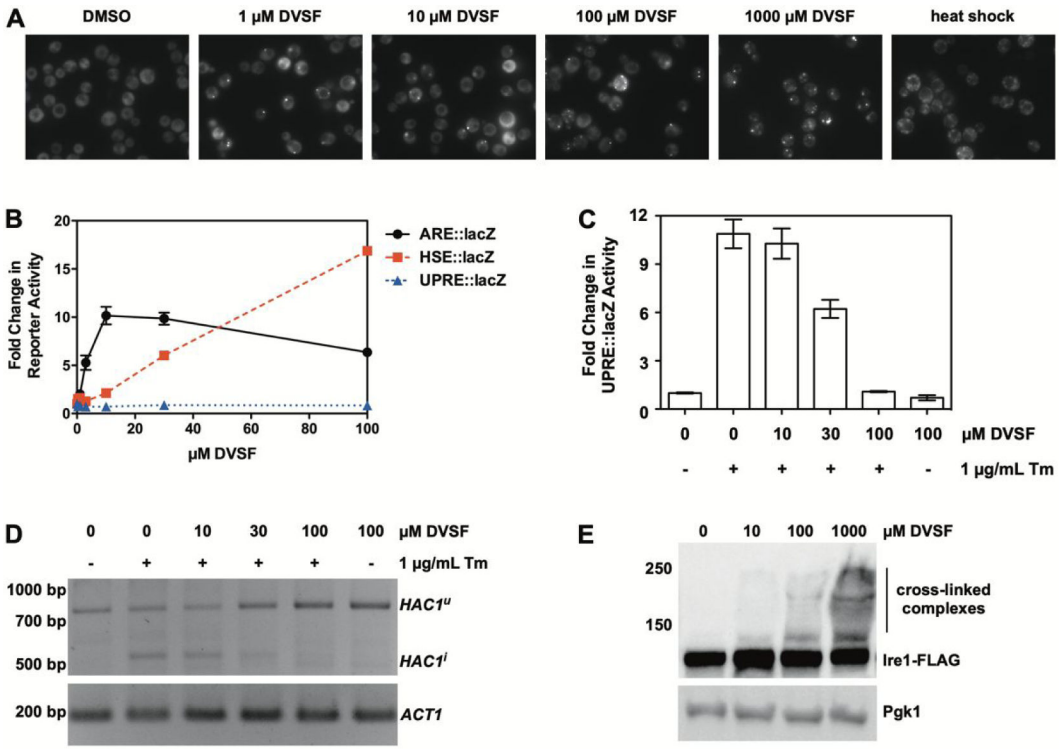

Figure 5. DVSF Activates Cytosolic Protein Damage Stress Responses but Inhibits ER Stress Signaling.

(A) Yeast cells expressing GFP-tagged Hsp104 were treated with indicated concentrations of DVSF or heat shock (at 42°C) for 15 min. Results are representative of three independent experiments. (B) Yeast transformed with constructs regulated by Yap1 (ARE::lacZ), Hsf1 (HSE::lacZ), or Hac1 (UPRE::lacZ) were treated with indicated concentrations of DVSF for 1 h at 30°C. β-Galactosidase activity was measured as described in the Materials and Methods. Results are the average ± standard error of the mean for six independent measurements. (C) Cells transformed with the UPRE::lacZ reporter were pretreated with the indicated DVSF concentrations for 30 min prior to treatment with 1 μg/mL Tm for 1 h. β-Galactosidase measurements show the average ± standard error of the mean for six independent measurements. (D) RNA was extracted from yeast treated with DVSF for 30 min prior to exposure to Tm for 30 min. RT-PCR was conducted to monitor *HAC1* splicing. *ACT1* levels were monitored as a loading control. (E) Cells expressing FLAG-tagged Ire1 were treated with increasing concentrations of DVSF for 1 h at 30°C. FLAG-tagged Ire1 was detected in protein lysates by immunoblotting. Pgk1 levels were monitored as a loading control. Results are representative of three independent experiments.

Table 1.

Identification of Proteins Associated with Eug1 and Pdi1 After Treatment of Cells with DVSF.

Biological Process	Eug1-Associated Proteins (55)	Pdi1-Associated Proteins (79)
Cell Wall Organization & Biosynthesis	Gas1, Bgl2, Exg1, Ecm14, Rot2, Cis3, Scw4, Uth1, Kre2, Gas3, Sim1, Scw10, Utr2, Ecm33, Cts1, Ygp1, Bar1, Kre5, Ktr4, Gas5	Gas1, Bgl2, Exg1, Ecm14, Rot2, Cis3, Scw4, Uth1, Kre2, Sun4, Gas3, Sim1, Scw10, Utr2, Ecm33, Cts1, Ygp1, Ktr7, Dfg5, Bar1, Kre5, Dcw1, Ktr3, Crh1, Gas5
Protein Folding	Pdi1, Sil1, Eug1, Scj1, Mpd1, Mpd2, Cpr5, Ero1, Lhs1, Kar2	Pdi1, Sil1, Eug1, Scj1, Mpd1, Mpd2, Cpr5, Ero1, Eps1, Erv25, Cpr2, Lhs1, Slp1, Fpr2, Kar2
Protein Glycosylation	Ktr1, Cwh41, Gtb1, Yos9, Mnl1, Wbp1	Ktr1, Cwh41, Gtb1, Yos9, Gda1, Mnl1, Mnn1
Protein Catabolism	Pep4, Prc1, Atg42, YDR415C, Ape3, Prb1	Pep4, Prc1, Atg42, YDR415C, Ape3, Cps1, Prb1
Lipid Catabolism	Plb1	Plb1, Gpi16, Plb2
Membrane Transport	Mid1	Adp1, Mid1, Fet5, Pma1
Other/Unknown	YNR066C, Tos1, Chc1, Rny1, YEL068C, YFR018C, Toh1, YCL049C, YHR202W, YJL132W, Pho3	YNR066C, Tos1, Vma1, Rny1, YCL049C YEL068C, Pho3, YFR018C, Pep1, Psg1, Vps62, Toh1, Pho11, YHR202W, YJL132W, Npc2, Faa4, Chc1

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