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Analysis of ER Resident Proteins in *S. cerevisiae*: Implementation of H/KDEL Retrieval Sequences

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Abstract

An elaborate quality control system regulates ER homeostasis by ensuring the fidelity of protein synthesis and maturation. In budding yeast, genomic analyses and high-throughput proteomic studies have identified ER resident proteins that restore homeostasis following local perturbations. Yet, how these folding factors modulate stress has been largely unexplored. In this study, we designed a series of PCR-based modules including codon-optimized epitopes and FP variants complete with C-terminal H/KDEL retrieval motifs. These conserved sequences are inherent to most soluble ER resident proteins. To monitor multiple proteins simultaneously, H/KDEL cassettes are available with six different selection markers, providing optimal flexibility for live-cell imaging and multicolor labeling *in vivo*. A single pair of PCR primers can be used for the amplification of these 26 modules, enabling numerous combinations of tags and selection markers. The versatility of pCY H/KDEL cassettes was demonstrated by labeling BiP/Kar2p, Pdi1p, and Scj1p with all novel tags, thus providing a direct comparison among FP variants. Furthermore, to advance *in vitro* studies of yeast ER proteins, *Strep*-tag II was engineered with a C-terminal retrieval sequence. Here, an efficient purification strategy was established for BiP under physiological conditions.

Keywords

BiP; Scj1p; Pdi1p; H/KDEL retrieval sequences; GFP variants; mCherry; pFA6a plasmid; UPR

Introduction

Approximately 30% of all newly synthesized proteins in *Saccharomyces cerevisiae* are delivered to their final destinations via the secretory pathway (1). Within this pathway, quality control is highly regulated in the endoplasmic reticulum (ER) thus supporting protein synthesis and maturation. The specialized milieu of the ER is composed of folding factors – commonly referred to as chaperones and foldases – that assist in the folding and modification of nascent or partially folded proteins. When protein abundance in this compartment exceeds its capacity due to various stimuli, the unfolded protein response (UPR) is activated. Factors leading to ER stress include alterations in calcium stores, oxidative stress or disturbances to the redox potential in the luminal environment, or an increase in protein synthesis rates that far surpass the folding rates of chaperones (as

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reviewed in (2, 3)). Yet, the ability of cells to respond to perturbations in ER function and to cope with various stressors is critical for cell survival. To alleviate ER stress, the UPR induces a broad transcriptional change, which results in the increased production of protein folding (4), degradation (5–7), and lipid (8) components, followed by the expansion of ER membranes (9, 10).

Select interactions of chaperones, co-chaperones, and foldases are prominent in ER translocation of nascent proteins, protein folding and maturation, karyogamy, and ER-associated degradation (ERAD) (11–15). Most, if not all, soluble ER resident proteins are transported continuously to the Golgi where they are then returned to the ER by retrograde transport machinery, as illustrated in Figure S1, Supplementary Material. In fact, Pelham and colleagues (16) demonstrated that an HDEL retrieval motif was both necessary and sufficient to retain proteins within the early secretory pathway. A similar trafficking mechanism was demonstrated for the KDEL retrieval sequence (17). This variation of residues potentially explains the divergence of C-terminal motifs detailed in Table 1. HDEL-dependent retrieval is required for cell growth in budding yeast (18) and the H/KDEL tetrapeptide must be located as the final four residues of recombinant proteins (16). Notably, the alteration of either the residue type or location will inhibit protein function (16, 18).

Although many ER-folding factors have been studied extensively, the full range of proteins contributing to cell homeostasis is unknown, and how they function together is poorly understood. Investigations of quality control mechanisms in the early secretory pathway require the development of *in vivo* and *in vitro* systems. Specifically in yeast, homologous recombination is a highly efficient approach that allows for a variety of gene modifications (19), including directed chromosomal integration of any tag fused to a protein expressed from its native promoter (20–22). In particular, the development of PCR-mediated techniques has facilitated rigorous genetic and biochemical analyses of *S. cerevisiae* genes. Numerous cassettes enabled the evaluation of protein function, interactions, and subcellular distribution in budding yeast (23, 24), although PCR-based modules were not utilized in the ER luminal environment. The ER lumen is unique among folding compartments in eukaryotes because it provides a sufficiently oxidizing environment for disulfide-linked protein folding, and foldases (e.g. Pdi1p, Ero1p, and Eug1p, Table 1) that promote the formation of disulfide bonds (9). In higher eukaryotes, Jain et al. (25) demonstrated the impairment of GFP maturation and lack of fluorescence within the ER lumen; however, fluorescent protein (FP) variants have not been evaluated thoroughly in the early secretory pathway of yeast (26).

In recent years, genomic analyses revealed the coordination of quality control mechanisms that maintain cellular homeostasis when compromised by external disturbances (4), and further characterized the functional interdependencies of genes required for protein folding in the ER (27). As a result of high-throughput technologies, evaluations of the *S. cerevisiae* proteome systematically identified protein interactions (28–31), localization (32, 33), abundance (1), and gene disruption phenotypes (34–37). Unfortunately by design, high-throughput technologies have not accommodated specific motifs required for ER protein function and intracellular location, therefore existing systems may not provide an accurate evaluation of ER resident proteins, as noted previously (33). To experimentally address the underlying mechanisms of ER homeostasis and cell physiology, the development of systems incorporating H/KDEL motifs are a prerequisite to assess spatial localization, complex interactions, and dynamics of ER folding factors.

In this paper, we describe a set of 26 plasmids containing 9 fluorescent proteins spanning the visible spectrum that can be used for multicolor imaging of ER resident proteins. FP variants, *c-myc* and HA epitope tags have been codon-optimized for yeast. To our

knowledge, this is the first study for which the yeast-enhanced (yE) *c-myc* and HA epitope tags maintain optimal sequences for *S. cerevisiae* (38) in conjunction with C-terminal retrieval motifs. To further promote the use of high-throughput technologies, the versatile affinity peptide, *Strep*-tag II, is also available with an N-terminal thrombin cleavage site and C-terminal HDEL motif. A detailed protocol is provided for the effective *Strep*Tactin purification strategy of BiP from its native environment when fused to *Strep*-tag II. Additionally, each cassette is available with *kanMX*, *hphMX4*, or *Streptoalloteichus hindustanus* bleomycin (*Sh ble*) gene in order to increase the repertoire of selection markers in *S. cerevisiae*, as well as one of the selective marker genes *TRP1*, *LEU2*, or *URA3* used with appropriate auxotrophic yeast strains. All pCY H/KDEL modules maintain primer homology consistent with established cassettes derived from pFA6a (23, 39–42). As such, the pCY H/KDEL cassettes series implements motifs essential for proper function and trafficking, enables PCR-based gene modification at the C-termini of chaperones and foldases, while significantly expanding the repertoire of available modules used to monitor protein dynamics and protein-protein interactions in yeast.

Results and Discussion

Versatile Cassette Design and Implementation

To differentiate the pivotal roles of ER resident proteins requires the design of functional probes that facilitate protein localization and interactions in an oxidized environment. Engineered FPs provide a diverse array of tools for biological imaging (43–46) and encompass techniques that evaluate protein dynamics (i.e. fluorescence recovery after photobleaching (FRAP) (47–50)), protein-protein interactions (i.e. fluorescence resonance energy transfer (FRET) (51–54)), and organelle continuity (i.e. fluorescence loss in photobleaching (FLIP) (55)). In contrast, the *Strep*-tag II affinity tag (56) coupled with effective procedures for detection, purification, and immobilization has resulted in the rapid isolation of recombinant gene products (57). In this study, conventional FPs and affinity tags were modified and select ER chaperones and foldases that assist in protein maturation, degradation, and export (Figure S1, Table 1) were evaluated.

To promote the utility of our PCR-based modules (Figure 1, Tables S1 and S2), cassettes were designed with several considerations. First, to identify protein complexes localized to the early secretory pathway, affinity and FP tags have been employed with native ER retrieval motifs – either an HDEL or KDEL sequence. Second, although GFP and DsRed variants result in well-separated spectra for multicolor imaging, an in-depth evaluation of FP variants in the ER lumen of *S. cerevisiae* has not been investigated (26). Previous studies suggested that exposed cysteines (e.g. C49 and C71) within the ER lumen may bind maturing GFP molecules or unfolded proteins containing cysteines, thus resulting in mixed disulfides. To prevent dimerization of fluorescent probes *in vivo*, monomeric versions of GFP variants utilize the A206R mutation (58). Consequently, yEmCFP and yEmCitrine (59) were incorporated in the pCY H/KDEL cassettes. Conversely, mCherry is a derivative of DsRed that contains no exposed cysteine residues (60). DsRed variants do fluoresce effectively in an oxidized environment – specifically the periplasm of *E. coli* (61). In this study, fluorophores for four-color imaging have been codon-optimized for yeast (59). With respect to cassette design, the introduction of a flexible linker between a target protein and tag is particularly advantageous, resulting in improved function (62) and purification (39). The implementation of a yeast-enhanced or codon-optimized linker encoding GFP variants resulted in a two-fold increase in fluorescent intensity in previous studies (59). Consequently, the pCY H/KDEL cassettes contain a yeast-enhanced polylinker, as well as FP variants and affinity tags codon-optimized for yeast.

Experimental Design and Assessment of H/KDEL Modules

The pCY H/KDEL cassette series promotes the facile modification of ER folding factors (Figure S1). Proteins whose modifications have been reported to compromise cell growth and activate cellular stress responses (e.g. UPR and heat shock response (HSR)) were considered ideal targets. A significant percentage of these proteins are characterized as essential (i.e. critical for cell survival) and are intimately associated with ER quality control (ERQC) (Table 1). However, their respective functions are quite diverse: ER translocation, protein folding and maturation, ERAD, COP II vesicle budding (i.e. trafficking from ER-to-Golgi, defined as anterograde transport), retrograde transport (i.e. trafficking from Golgi-to-ER), and N- and O-linked glycosylation.

ER resident proteins are generally acknowledged to be up-regulated following UPR induction (4). As summarized in Table 1, the UPR regulates genes of the early secretory pathway to varying degrees. Of the six canonical UPR genes – *KAR2*, *EUG1*, *PDII*, *LHS1*, *FKB2*, and *ERO1* (4, 63, 64) – the effects of ER resident proteins, BiP/Kar2p and Pdi1p, fused to FP variants and affinity tags were evaluated in this work.

Commonly referred to as BiP, Kar2p is the yeast homolog of binding protein immunoglobulin BiP (also known as Grp78 (65)). As one of two distinct Hsp70 molecular chaperones of the ER lumen, BiP is conserved throughout eukaryotes and binds preferentially to hydrophobic residues of nascent or unfolded proteins (66, 67). Although a resident of the ER lumen, *S. cerevisiae* BiP/Kar2p traffics to the cis-Golgi where it is retrieved due to its C-terminal HDEL tetrapeptide and direct association with Erd2 (68, 69). Endogenous BiP is essential for cell growth and strain viability (70) and maintains a molecular weight of 74.5 kDa. Similarly, protein disulfide isomerase (Pdi1p) is a ER resident protein conserved in all eukaryotes, required for cell growth (71), and contains an HDEL retrieval motif. Pdi1p functions as a catalyst by promoting the formation, reduction, and isomerization of disulfide bonds in nascent proteins (72, 73), and maintains a molecular weight of 58.2 kDa. These attributes provide a unique experimental system to evaluate recombinant BiP and Pdi1p expressed under the regulation of their native promoter in *S. cerevisiae*.

Our research interests focus on the spatiotemporal dynamics of ERQC and protein trafficking within the early secretory pathway; thus, we investigated the effects of fluorescent proteins variants and affinity tags fused to ER resident proteins - BiP, Pdi1p, and Scj1p – in multiple strains. *S. cerevisiae* strains BJ5464 (*MATα ura3-52 trp1 leu2Δ his3Δ 200 pep4::HIS3 prb1-Δ 1.6R can1 GAL*, ATCC® 208288™, (74)), BY4742 (*MATα his3Δ 1 leu2Δ 0 lys2Δ 0 ura3Δ 0*, EUROSCARF Y1000, (75)), W303 (*MATα ade2-1 ura3-1 his3-11 trp1-1 leu2-3 leu2-112 can1-100*, ATCC® 208353™, (76)) and derivatives listed in Table 2 were used in this study. Yeast strain BJ5464 lacks efficient vacuolar degradation due to the deletion of *PEP4* and *PRB1* and is predominantly used for heterologous protein expression. In contrast, BY4742 is typically used to evaluate endogenous cellular processes and protein function, while numerous studies have examined UPR initiation and attenuation in W303.

Implementation of the HDEL Retrieval Sequence: Expression, ER Localization, and Trafficking Effects

The expression of BiP-FP fusions – incorporating blue, green, yellow, and red variants designed with the C-terminal HDEL retrieval sequence – was assessed by a facile and direct approach. As previously described (77), an in-gel fluorescent technique enabled the discrimination of the intrinsic fluorescence of FP variants when fused to the ER translocon, Sec61, a membrane protein whose C-terminus is exposed to the cytosol. We exploited the

spectral characteristics of FP variants expressed in the oxidized environment of the ER in order to examine BiP fusions whose excitation and emission profiles constitute the entire spectrum of visible light. As shown in Figure 2A, in-gel fluorescent methods also provide an adequate means of examining BiP-FP fusions, as compared to parental strains.

To confirm the expression of desired products, recombinant proteins were probed using anti-GFP via standard western blot techniques. Protein concentrations were normalized to an established loading control, β -actin, which provides a means of direct comparison among various fusion proteins (Figure 2B). Notably, the parental strain of yeast had a profound effect on the concentration (i.e. molecules/cell) of BiP-FP when observed at an identical stage of growth. This difference was most likely a result of the degree to which ERQC mechanisms were initiated in various parental strains. Protein levels of BiP are increased during cell stress as a consequence of transcriptional up-regulation following UPR or HSR activation. We established that the UPR is initiated earlier in BJ5464 cells – the strain lacking vacuolar proteases essential for the degradation of aberrant proteins – as compared to BY4742 by implementing classical UPR sensors (4, 78). A greater magnitude of the response (i.e. BiP protein levels and fluorescence of the UPR sensor) was observed in this protease-deficient strain (data not shown). Data in Figure 2 are consistent with these results.

Previously, the oxidative environment of microbial and eukaryotic cells has resulted in either a lack of fluorescence (79), oligomerization (25), or mixed disulfides (80) for FP fusions. We evaluated all fluorescent cassettes within the ER by in-gel and western blot analyses (Figure 2C, D) under non-reducing conditions to examine the possibility of oligomerization or disulfide formation. As shown, all strains that recombinantly expressed BiP-FP fusions displayed one product regardless of whether the probe of interest was a GFP variant, a designed monomer (e.g. yEmCFP or yEmCitrine that contains the A206R substitution to prevent dimerization), or mCherry (DsRed variant) that does not contain cysteines exposed to the lumen.

Growth rates were calculated for BiP fused to Cerulean, Venus, or mCherry as an indirect measure of whether FP-fusions adversely affected cell physiology. Since previous studies by Townsley et al. (18) demonstrated that modification of the ER retrieval sequence compromised strain viability, a characteristic generation time of each FP fusion was compared to *S. cerevisiae* strain BJ5464 (i.e. wildtype). In Figure 3A, the mean optical density (OD_{600}) is shown for each time point sampled and error bars depict the minimum and maximum measurement from three experimental replicates. The semi-log plot of Figure 3A shows that the growth rate of BiP-Cerulean and BiP-mCherry is slower than either BiP-Venus or the parental strain. Two-sample t-tests statistically confirmed this hypothesis. As shown in Figure 3A (inset), vertical bars represent the mean doubling time for each strain whereas the corresponding p-value is located above each bar. Error bars are defined as the 95% confidence interval of the mean. At the 5% significance level ($\alpha = 0.05$), the mean doubling time of BiP-Venus (99.7 min) was equal to the wildtype strain, BJ5464 (102 min). In contrast, the doubling times of BiP-Cerulean (133 min) and BiP-mCherry (122 min) were greater than the control. Interestingly, when time course experiments were performed for BiP fused to yeast-enhanced variants (e.g. yEmCFP, yEmCitrine, and yEGFP implementing the codon-optimized polylinker), doubling times of both monomeric variants were comparable to the wildtype strain. Two-sample t-tests confirmed the equality of mean doubling times for wildtype, yEmCFP, and yEmCitrine strains (Figure 3B, inset). Although these results indicate minor perturbations to the system, increased doubling times do infer some impact on the cellular physiology when BiP was fused to Cerulean, mCherry, or yEGFP.

The ER localization of endogenous BiP was determined originally by immunofluorescence techniques using anti-Kar2p primary antibodies, as published by Rose and colleagues (65). To correlate the intracellular localization of BiP fused to FP variants in several *S. cerevisiae* strains, we first probed for the endogenous species using anti-Kar2p (81). As depicted in Figure 3C, immunofluorescence of all parental strains defined three ER subcompartments (i.e. perinuclear ER, cortical ER, and tubular connections) when imaged by confocal microscopy. Furthermore, live-cell imaging of FP variants did not cause mislocalization. By comparison to the immunofluorescent images, it is apparent that all BiP-FP fusions were correctly localized to the ER, as demonstrated in Figure 3D. In contrast to immunofluorescent techniques, the implementation of BiP-FP variants with retrieval motifs provides a distinct advantage as it facilitates time-lapse imaging, enabling real-time protein monitoring during cell homeostasis and immediately following disturbances to the system (e.g. activation of ER stress). Live-cell images of BiP-FPs assessed during early- to mid-log phase depicts a spatial distribution vastly similar to immunofluorescence. However, under conditions of nutrient deprivation during stationary phase, (>1.8 OD's) BiP distributes to form larger inhomogeneities within the ER, a phenomenon that has been assessed previously for UPR complexes (82, 83). FRAP experiments (Video S1, Supplemental Material) showed that intracellular BiP clusters are localized to the ER based upon the recovery of fluorescence within the region of interest (ROI) photobleached.

Within the early secretory pathway of *S. cerevisiae*, Pelham and colleagues (84) established that the HDEL recognition system can become saturated. Previous studies confirmed that endogenous BiP is secreted during overexpression of an HDEL-tagged prepro-alpha factor. To ensure that the retrieval motif of our cassette series is recognized and that retrograde transport machinery is not overwhelmed, time course experiments were performed for all BiP-FP variants. When cultures attained log-phase growth, the media was probed for BiP by standard western blot techniques. Our results indicated that BiP was not secreted in the wildtype strain. Interestingly, for BiP-FP fusions expressed under the regulation of the native *KAR2* promoter, no detectable secretion was observed (data not shown). Collectively, these results confirm that the HDEL receptor effectively recognized modules containing the retrieval motif.

The utility of our cassettes was further validated when applied to the ER foldase, Pdi1p. All FP variants containing C-termini HDEL retrieval sequences were directed to the *PDI1* ORF. Similarly, methodologies described above were completed for Pdi1p. In Figure 4A, each Pdi1p-FP fusion was distinguishable via an in-gel fluorescent technique. Western blot analysis verified the expression of fusion proteins derived from GFP (Figure 4B). In order to normalize each species for direct comparison, a subsequent analysis probed for the Tubulin loading control. Of particular note is the decreased fluorescence intensity of blue variants (Figure 4A) compared to the results of the GFP primary antibody and established loading control (Figure 4B). Similarly in Figure 4A, the fluorescence intensity of Pdi1p-mCherry-HDEL expressed in BY4742 was significantly less than its intensity in BJ5464, which is a direct result of total protein concentration probed by anti-Tubulin. The decreased recovery of BY4742 Pdi1p-mCherry-HDEL may be partially due to different growth rates of these parental strains. In contrast to our investigations of BiP-FP – specifically the lack of degraded FP variant, mixed disulfides, or oligomerization – Pdi1p-Venus does contain a fluorescent degradation product. Effectively, 36 % of total recombinant Pdi1p expressed is degraded and detected via in-gel fluorescence and western blot analysis (data not shown).

Although live-cell imaging adequately detected FP variants yEGFP, Venus, yECitrine, and mCherry during the live-cell imaging of recombinant Pdi1p (Figure 4C), additional fluorescent aspects are noteworthy. For example, the intensity of blue GFP variants yECFP and yEmCFP was not detected prominently via confocal microscopy (Figure 4D) under the

parameter settings described in *Materials and Methods*. These results are in agreement with the decreased fluorescence intensity of blue variants, as examined by in-gel fluorescent techniques and confirmed by western blot procedures (Figure 4, A & B). We have previously demonstrated the significance of the increased sensitivity provided by gallium arsenide-phosphide (GaAsP) detectors (85), which are two-fold more sensitive than standard PMTs (86). Thus, the use of Pdi1p fused to either yECFP or yEmCFP reveals a dramatic limitation for experiments involving time-lapse imaging. The degradation of GFP variants was observed in the vacuole of green, yellow, and blue FPs fused to Pdi1p (Figure 4C); however, DsRed variants are not detected appreciably within the vacuole when expressed as a Pdi1p-FP fusion.

KDEL Retrieval is used for ER Co-chaperone, Scj1

Interestingly, the mammalian homolog of BiP (i.e. Grp78) contains a C-terminal KDEL retrieval sequence even though the receptor actually binds the tetrapeptide HDEL more tightly (87, 88). Pelham and colleagues (16) determined varying affinities of retrieval sequences (i.e. FEHDEL, SEKDEL, SAEARRL, etc.) for retrograde transport machinery. Hence, there exists some variation in the first two positions of the motif, which has been proposed to affect the rate of retrieval amongst resident proteins (89) (Table 1). In *S. cerevisiae*, only Scj1p – an ER luminal co-chaperone involved in protein folding and maturation – contains this KDEL motif. To determine the impact of trafficking sequences, we implemented a C-terminal MLKDEL sequence and investigated Scj1 FP fusions. In-gel fluorescence distinguished between parental strains and the recombinantly expressed Scj1p FP variants (Figure 5A). Our analyses with Scj1p indicated that in-gel fluorescent methods are an adequate means of assessing protein levels that are significantly less than mM concentrations in the luminal environment. In Figure 5B, western blot analysis confirmed the expression of GFP variants. To normalize each species for direct comparisons, subsequent analysis probed for the loading control, Actin. To our knowledge, Scj1p has not been analyzed *in vivo*; thus, live-cell imaging techniques of Figure 5C demonstrate the successful trafficking of Scj1p within the early secretory pathway of yeast. We have confirmed these results via examination of three common strains. Images (Figure 5C) suggest that Scj1-FP does traffic to the vacuole whereas in-gel fluorescent analyses detect a low percentage (i.e. 28 %) of degraded FP variant, as compared to total expression of Scj1p-FP. Of particular note during live-cell imaging, we utilized GaAsP detectors in order to visualize endogenous levels of Scj1p FP fusions. This experimental condition may limit the utility of this construct for proposed experiments involving time-lapse microscopy.

H/KDEL motifs: A Comparative Study

Time course experiments were performed in triplicate for BiP-Venus-HDEL, Pdi1p-Venus-HDEL, and Scj1p-Venus-KDEL within the same parental strain, BJ5464. Figure 5D shows the mean optical density at each time point, where error bars represent the minimum and maximum measurement. The mean doubling time was calculated for each strain and is represented by the vertical bars of Figure 5D (inset). Error bars are defined as the 95% confidence interval of the mean. Two-sample t-tests evaluated the equality of the means for the wildtype strain, BJ5464, compared to BiP, Pdi1p, or Scj1p fusion proteins. The mean doubling time of all four strains was statistically equal as denoted by the corresponding p-values. Thus, the utilization of pCY H/KDEL modules has been sufficiently corroborated for a ER molecular chaperone (BiP), co-chaperone (Scj1p), and foldase (Pdi1p).

Strep-tag II Purification of an ER Chaperone

In addition to FP variants and epitope tags, small affinity peptides enable the rapid isolation of recombinant gene products when coupled with effective procedures for detection, purification, and immobilization (57). *Strep-tag II* is a versatile affinity peptide that does not

impede protein translocation nor inhibits protein-folding events, and is largely resistant to cellular proteases (90). As compared to the original *Strep*-tag (i.e. WRHPQFGG, (91)), *Strep*-tag II permits greater flexibility in its choice of attachment site (56) and is used with an engineered variant of streptavidin, *Strep*Tactin, which has an improved peptide binding capacity (92). These attributes strongly advocate the implementation of *Strep*-tag II to detect and purify ER folding factors.

To characterize the biochemical activity of BiP and Pdi1p, existing purification strategies employ either multistep purification methods (i.e. utilizing the affinity of a BiP/Kar2p specific antibody combined with hydroxyapatite column chromatography (93) and isolation of endogenous Pdi1p in *S. cerevisiae* via precipitation followed by affinity chromatography (94)) or strong denaturing conditions (e.g. N-terminal His₆-tagged BiP expressed in *E. coli* and purified by Ni²⁺-NTA affinity chromatography (95)). In contrast, we incorporated an eight-amino acid yeast-codon optimized peptide (WSHPQFEK) referred to as *Strep*-tag II (56), and utilized the *Strep*Tactin affinity method.

Prior to this study, *S. cerevisiae* ER resident proteins BiP and Pdi1p were detected solely by standard techniques, using primary antibodies derived from the endogenous gene. It is often desirable to detect *Strep*-tag II fusions with high sensitivity – either by standard immunoblotting techniques, immuno-fluorescence, or flow cytometry – for which conjugated *Strep*Tactin/enzyme substrates are commercially available (90). Alternatively, monoclonal antibodies such as *Strep*MAB-Classic (IBA BioTAGnology) permit the sensitive detection of fusion proteins. In Figure 6A, *Strep*MAB-Classic was used to probe for the expression of BiP-*Strep*-tag II - HDEL, which was detected at the predicted molecular weight of 76.9 kDa. As illustrated in Figure 6B, the cell lysate, which consists of recombinantly expressed BiP and host proteins, was immobilized to *Strep*Tactin. Non-specifically bound proteins were removed by sequential washes. The desired product was then eluted under mild conditions, via competition with D-desthiobiotin. In Figure 6C, the results of an efficient BiP-*Strep*-tag II purification strategy are shown. Approximately 80 OD₆₀₀ (equivalent to 80 × 10⁷ cells) yielded a 54% recovery of total BiP from the lysate (eluates 1 – 5), while less than 1% of product remained bound to the resin. Additionally, 34% of the product resided in the flow-through thereby providing a basis from which to optimize future studies.

Applications of pCY H/KDEL cassettes

The molecular mechanisms underlying ER homeostatic regulation have been identified, but how they coordinate the comprehensive restructuring of ER resident proteins via transcriptional regulation and remodeling of organelle morphology is only partially understood. To experimentally address these questions, we have designed and implemented a new series of versatile PCR-based modules for C-terminal tagging of ER resident proteins in yeast. Collectively, we established that in-gel fluorescence is an efficient method to evaluate recombinantly expressed ER resident proteins fused to FP variants, prior to confirmation by western blot analyses. Moreover, live-cell imaging techniques affirmed the correct localization of BiP, Pdi1p, and Scj1p fusion proteins. The viability of each novel strain expressing recombinant fusions was quantified by time course experiments, from which doubling times were statistically evaluated. Neither Pdi1p fused to blue variants nor Scj1p fused to yellow variants possessed adequate fluorescence for time-lapse imaging; therefore, we have identified limitations associated with the choice of FP in the ER luminal environment. We successfully demonstrated that affinity purification was achieved for BiP-*Strep*-tag II - HDEL under physiological conditions. Combined, the pCY H/KDEL cassettes enable the measurement of protein concentrations compatible with western blot analyses, utilize optimal fluorescence spectra and selection for multicolor labeling *in vivo*, facilitate

protein trafficking studies in the secretory pathway, and investigate select protein interactions.

We envision this series of modules to be an invaluable tool, broadly applicable, to yeast researchers investigating the role of ER resident proteins in cell homeostasis, ER-to-Golgi trafficking, protein structure/function relationships of purified ER resident proteins, and the kinetics of ER molecular chaperone/co-chaperone interactions. Interestingly, the implementation of H/KDEL modules could effectively sense the activation of cellular quality control mechanisms *in vivo*, similar to results achieved by existing UPR sensors fused to GFP or DsRed variants (4, 78) and recently demonstrated by Lajoie et al. (96). Prolonged UPR activation is linked to pathophysiological processes, as well as a number of prevalent diseases (reviewed in (97, 98)) highlighting UPR modulation as an emerging focus of therapeutic design (99). Notably, many components of neurodegenerative and hereditary diseases have been studied in *S. cerevisiae* (100–103), thus advocating the use of this cassette series in a model eukaryote to investigate cell physiology and disease.

Materials and Methods

Construction of Plasmid Cassettes

Design details associated with the pCY H/KDEL cassette series are summarized in Table S1. Similarly, key components of these versatile modules are depicted in Figure 1. A comprehensive overview of all available cassettes - identifying the tag, retrieval sequence, and selection marker - is provided in Table S2. Corresponding oligonucleotides synthesized by Integrated DNA Technologies, Inc. (Coralville, IA) are listed in Table S3.

All cassettes maintain conserved forward and reverse primer homology consistent with established plasmids (59). Plasmids pBS7, pBS10, and pBS35 ((51), Yeast Resource Center, University of Washington) derived from pFA6a-GFP(S65T)-kanMX (42) or the pCY series of cassettes (77) were used as the parental vectors for all modules identified in Table S1. Residue mutations of yeast codon-optimized GFP variants have been summarized elsewhere ((77), in Supplemental Table S4). In many cases, a two-step ligation process was essential. First, the fluorescent protein or epitope tag was amplified with a desired retrieval sequence and inserted into the base construct at the *Bam*HI and *Bss*HII restriction sites. *S. cerevisiae* codons corresponding to the final six amino acids of BiP/Kar2p (i.e. FEHDEL) or Scj1p (i.e. MLKDEL) were implemented as ER-retrieval sequences (Figure 1B), referred to as HDEL and KDEL (e.g. H/KDEL). A codon-optimized polylinker was then inserted at the 5' end of select tags using *Xma*I and *Pac*I (Figure 1, A & B). To determine the appropriate construct to be sequenced (DNA Sequencing Facility, University of Pennsylvania), the polylinker was designed with a unique *Af*III site (Table S3). As a result of molecular engineering design, the first generation of recombinant fusion proteins required DNA amplification of the tags using forward primer HFR1 (Table S3). Forward primer HFR2 is essential when including the codon-optimized polylinker, which is conserved in all cassettes (Table S3, Addgene) provided to the non-profit plasmid repository Addgene (<http://www.addgene.org>).

As shown in Figure 1D, E and defined in Table S3, two complementary oligonucleotides encoding yeast-enhanced *c*-myc and HA epitope tags, as well as *Strep*-tag II (i.e. W-S-H-P-Q-F-EK, (56)) contain a 5' *Bam*HI and 3' *Bss*HII site. The implementation of *Strep*-tag II results in an efficient purification strategy complete with a thrombin cleavage site (i.e. LVPR[^]GS), where the cleavable recognition site is denoted by (^) and remaining residues are underlined (Figure 1E). The annealed DNA fragment was incubated with pBS7, pBS10, or pBS35 restricted vector and ligated with T4 DNA ligase (New England Biolabs) per manufacturer's instructions.

Escherichia coli strain DH5 α and standard techniques were used for DNA manipulations (104). DNA fragments were excised from agarose gels and purified by Zymoclean™ Gel DNA Recovery kit (Zymo Research) or Wizard® SV Gel and PCR Clean-up System (Promega). Preparation of plasmid DNA was completed using Zyppy™ Plasmid Miniprep kit (Zymo Research) or Wizard® Plus SV minipreps DNA Purification System (Promega).

Yeast Strains, Growth Conditions, and Validation

Yeast strains and derivatives listed in Table 2 were used in this study. Yeast were transformed using a modified version of the Gietz and Woods' *High-Efficiency Transformation Protocol* (105) provided by the Yeast Resource Center (University of Washington).

C-terminal fusion proteins expressed from their endogenous promoters were created by amplification of select modules and ultimately targeted to the *KAR2* (BiP), *PDI1*, or *SCJ1* ORF by homologous recombination (Figure 1F, Table S3). pCY H/KDEL modules were amplified using the Expand Long Template PCR System (Roche) and Buffer 3 under conditions provided (http://depts.washington.edu/yeastrc/pages/plasmids_protocols.html) with the exception that the annealing temperature for all PCR reactions was 57°C.

Similar to the pCY series of cassettes (77), a total of 6 genes were utilized for selection. Details are provided in Tables S1 and S3. To select for antibiotic-resistant transformants, the following concentrations were added to standard YPD plates when the media reached a temperature below 60°C: *kanMX*, geneticin® (G418, Gibco), 200 μ g/mL; *hphMX4*, hygromycin B in PBS (invitrogen™), 300 μ g/mL; and zeocin resistant gene *Sh ble*, Zeocin™ (invitrogen™), 200 μ g/mL. Alternatively, the selection of fusion proteins based on *TRP1*, *LEU2*, or *URA3* genes requires appropriate *S. cerevisiae* strains (e.g. *leu2 Δ 0* and *ura3 Δ 0*), such as those derived from Brachmann et al. (75). Resultant transformants were selected on synthetic complete (SC) plates lacking tryptophan, leucine, or uracil, as previously described (106).

Recombinantly expressed ER-resident proteins were confirmed by immunoblotting and genomic PCR. Genomic DNA was extracted using MasterPure™ Yeast DNA Purification kit (EPICENTRE® Biotechnologies) and amplified with primers listed in Table S3. Correct sequencing of genomic DNA was also confirmed for a fraction of novel strains.

In-gel Fluorescence

The intrinsic fluorescence of GFP and DsRed variants - designed with C-terminal H/KDEL retrieval sequences - was used in preliminary assays to examine the chromosomal integration of FP variants. *S. cerevisiae* strains that constitutively expressed BiP, Pdi1p, and Scj1p FP fusions were inoculated at an optical density (OD₆₀₀) of 0.1 in 5 mLs of YPD media (30°C, 275 rpm). Liquid cultures achieved early- to mid-log phase following 8 hrs. For BiP and Pdi1p green and yellow fluorescent variants, a volume equivalent to 1.0 OD₆₀₀ of cells was removed and centrifuged for 1 min at 13,000 $\times$ g. Likewise, a volume equivalent to 2.0 or 2.5 OD₆₀₀ of cells was removed for BiP and Pdi1p mCherry fusions and 5.0 or 2.5 OD₆₀₀ of cells were acquired for BiP and Pdi1p blue fluorescent variants, respectively. The protein concentration of endogenous ER co-chaperone Scj1p was expected to be less than BiP and Pdi1p, whereas Pdi1p constitutes ~ 2% of the protein in the ER (73). Thus an increased volume, equivalent to 5 OD₆₀₀ of cells, was removed for Scj1p-Venus and yEmCitrine fusions, then centrifuged for 1 min at 13,000 $\times$ g.

A detailed lysis protocol has been described (81). Here, lysate was combined with 3 $\times$ SDS loading buffer (150 mM Tris, pH = 6.8; 0.25 mg/mL bromophenol blue; 6% SDS; 30% glycerol) and proteins were resolved by SDS-PAGE. Gels were then scanned (Typhoon

9400 Variable Mode Imager, Amersham Biosciences) at excitation wavelengths of 457 nm (520BP40 filter), 488 nm (520BP40 filter), or 532 nm (580BP30 filter). A fluorescent molecular weight ladder at 488 nm (BenchmarkTM Fluorescent Protein Standard, invitrogenTM) was used as a standard.

Western Blot Analyses

Yeast extracts were prepared as described in the previous section, and then heated at 100°C in the presence of dithiothreitol (DTT). Under non-reducing conditions, a similar protocol was followed in the absence of DTT. Proteins were resolved by SDS-PAGE, electrotransferred overnight (18 V at 4°C) to Trans-Blot[®] Transfer Medium pure nitrocellulose membrane (Bio-Rad), and probed by standard western blot procedures (107). Primary antibodies rabbit anti-Kar2p (polyclonal antibody, 1:10000, A. Robinson lab, (81)), rabbit anti-GFP (polyclonal antibody, ab6556, 1:1700 (e.g. Scj1p GFP variants) or 1:5000 (e.g. Pdi1p GFP variants), abcam[®]), mouse anti-β-Actin (monoclonal – loading control, ab8224, 1:2000, abcam[®]), rat anti-Tubulin (monoclonal [YL½] – loading control, ab6160, 1:1:5000, abcam[®]), or mouse anti-*Strep*-tag[®] II (*Strep*MAB-Classic, IgG1, 2-1507-001, IBA BioTAGnology, 1:2500) were followed by incubation with secondary antibodies Alexa Fluor[®] 633 goat anti-rabbit IgG (H+L) (invitrogenTM), Alexa Fluor[®] 488 goat anti-mouse IgG (H+L) (invitrogenTM), and Alexa Fluor[®] 488 goat anti-rat IgG (H+L) (invitrogenTM) at 1:1700 – 1:5000, 1:1400, and 1:2500 dilutions, respectively. Alternatively, the ECLTM Prime Western Blotting Detection reagent (GE Healthcare, AmershamTM) was used in the presence of 1:2000 anti-rabbit IgG, Horseradish Peroxidase linked whole antibody (GE Healthcare, AmershamTM). Blots were then scanned (Typhoon 9400 Variable Mode Imager, Amersham Biosciences) and analyzed at the desired settings: 457 nm excitation wavelength and filter 529BP40, 488 nm excitation wavelength and filter 520BP40, or 633 nm excitation wavelength and filter 670BP30.

Spectroscopic Studies: Confocal Microscopy

Immunofluorescence—Endogenous BiP was probed by immunofluorescence with an anti-Kar2p polyclonal antibody in all parental strains (Figure 3C). *S. cerevisiae* parental strains BJ5464, BY4742, and W303 were cultured overnight in 5 mLs of YPD media (30°C and 275 rpm) to attain early- to mid-log phase ($0.3 < OD_{600} < 1.2$). Yeast cells were fixed and stained for immunofluorescence without dehydration as described (108), a modified version of the protocols established by Rose (109) and Pringle et al. (110). A volume equivalent to 1 OD₆₀₀ of cells was centrifuged at $8,000 \times g$ for 1 min, rinsed and resuspended in PBS (Phosphate Buffered Saline, 1.44 g/L Na₂HPO₄, 0.2 g/L KCl, 0.24 g/L KH₂PO₄, 8 g/L NaCl, pH=7.35), then fixed with 4% paraformaldehyde (EM grade paraformaldehyde, Electron Microscopy Sciences) for 30 min at room temperature. Cells were washed with appropriate buffers, as described (108), and treated with Zymolyase (a volume equivalent to 100T, Zymo Research) in combination with 2 μL of β-mercaptoethanol (SIGMA-ALDRICH[®]) at 30°C for 20 min. Cells were rinsed with 40 mM KPO₄ (pH = 7.35), 500 μM MgCl₂, and 1.2 M sorbitol (108) and immobilized for 30 min on coverslips (High-performance No. 1.5, Carl Zeiss) coated with poly-D-lysine (0.1% final concentration, MW > 300,000, SIGMA-ALDRICH[®]) and separated by a hydrophobic barrier (Aqua-Hold, Scientific Device Laboratory). Immobilized cells were incubated with blocking reagent (3% Bovine Serum Albumin (Fisher Scientific), 0.5% Tween 20 (BioRad) in PBS, pH = 7.35) for 1 hr at room temperature. Endogenous BiP was probed by the primary antibody rabbit anti-Kar2p (polyclonal antibody, 1:800, A. Robinson lab, (81)) and secondary antibody Alexa Fluor[®] 633 goat anti-rabbit IgG (H+L) (1:500, invitrogenTM) following 1 hr incubation at room temperature. Samples were washed repeatedly with blocking reagent, immersed in antifade reagent *SlowFade*[®] Gold (invitrogenTM), sealed and stored overnight at 4°C, then imaged the following day.

Images were obtained on a Zeiss LSM 780 confocal microscope (100×/NA 1.46 objective) under the following parameter settings: excitation at 633 nm, 16 line averages, and MBS 458/561/633 beam splitter. Images were further processed with Zen 2009 Light Edition (Carl Zeiss, Inc.).

Live-cell Imaging—Imaging of all strains recombinantly expressing BiP, Pdi1p, and Scj1p fluorescent fusion proteins was performed on a Zeiss LSM 780 confocal microscope (100×/NA 1.46 oil immersion objective). Growth conditions were identical to those described previously (77). When mid-log phase was achieved, 1 mL of culture was removed, centrifuged at $8,000 \times g$ for 1 min, and rinsed twice with SC media. Cells were then embedded in 2% agarose (SeaPlaque® GTG® Agarose, LONZA), dissolved in SC media, and coated with VALAP (1 vaseline: 1 lanolin: 1 paraffin).

BiP and Pdi1p fused to yEGFP were excited at 488 nm and imaged by 16 line averages on the Zeiss LSM 780 at 498 – 568 nm emission using a MBS 488/561 beam splitter and GaAsP detector. Likewise, yellow variants of all three protein fusions were excited at 514 nm and imaged by 16 line averages under the following settings: 516 – 594 nm emission, MBS 458/514 beam splitter, and GaAsP detector. It is important to note that the laser intensity was significantly increased during the imaging of Scj1p (e.g. excitation of Venus at 10 %) compared to either BiP (e.g. excitation of Venus at 1.2 %) or Pdi1p (e.g. excitation of Venus at 4.0 %) with similar gains. Due to the increased sensitivity of its GaAsP detectors, the Zeiss LSM 780 confocal microscope adequately captured the intensity of co-chaperone Scj1 fusion proteins when compared to conventional confocal microscopes equipped with standard photomultiplier tubes (PMTs). Live-cell images of BiP and Pdi1p mCherry fusions were assessed on the Zeiss LSM 780 at the following parameter settings: excitation at 561 nm, 16 line averages, 577 – 691 nm emission, and MBS 458/561 beam splitter. Images were further processed with Zen 2009 Light Edition (Carl Zeiss, Inc.).

FRAP was performed on BJ5464 BiP-yEGFP-linker-yEGFP-HDEL under conditions of saturated growth ($OD_{600} > 1.8$). A ROI (red circle, Video S1, Supplemental Material) was selected and photobleached resulting in total fluorescence of the ROI that was decreased to less than 55%. As shown, the ROI recovers fluorescence during the ~6.5 min time series, confirming the continuous structure of the ER, despite the regions of inhomogeneity. Video S1 was compressed to 4 fps.

Time Course Evaluations of Recombinantly Expressed ER Resident Proteins

Time course experiments were completed for cultures of BiP, Pdi1p, and Scj1p FPs and doubling times were compared to parental *S. cerevisiae* strains (i.e. wildtype). Strains identified in Figure 3A, B and Figure 5D were cultured overnight in 30 mLs of YPD media (30°C and 275 rpm), while attaining early- to mid-log phase ($OD_{600} \sim 1.5$) the following day. A starting culture was inoculated at 0.1 OD_{600} (total volume of 300 mLs YPD), which required a volume equivalent to 30 OD_{600} of cells. Cells were centrifuged for 8 min (4°C and $3,000 \times g$) and resuspended in 300 mLs of YPD. Every two hours, the optical density was recorded and a volume equivalent to 1.0 OD_{600} of cells was removed, followed by centrifugation at $8,000 \times g$ for 1 min. These samples were immediately rinsed with PBS (Phosphate Buffered Saline, 1.44 g/L Na_2HPO_4 , 0.2 g/L KCl, 0.24 g/L KH_2PO_4 , 8 g/L NaCl, pH=7.35) and centrifuged ($8,000 \times g$ for 1 min). Supernatant was discarded and cells were stored at –80°C.

Statistics

In order to evaluate whether the mean doubling times of recombinant strains were comparable to wildtype BJ5464, we first examined whether the growth rate of this parental

strain was equivalent to published reports. Di Talia et al. (111) verified that the characteristic generation time of *S. cerevisiae* S288C was 99 ± 1 min when cultured in rich medium, a value originally established by Warner (i.e. $t_d = 100$ min, (112)). Here, time course experiments were completed as replicates ($N = 10$) for BJ5464, resulting in a calculated mean doubling time (i.e. t_{d_BJ5464}) that was compared to the established growth of 99 min. A one sample, two-sided t-test confirmed that $t_{d_BJ5464} = 99$ min, at the 0.05 significance level. Thus, the control $t_d^* = 99$ min represents the mean doubling time of BJ5464 and corresponding 95% confidence interval (98.73, 105.34) under experimental conditions.

Growth rates of individual *S. cerevisiae* strains recombinantly expressing BiP, Pdi1p, and Scj1p were calculated by time course experiments performed in triplicate. For each time course performed, the doubling time was calculated. Under these scenarios, two-sample t-tests were completed for each strain, where the null hypothesis examined the equality of the means for wildtype, BJ5464, compared to BiP, Pdi1p, or Scj1p fusion proteins, at the 0.05 significance level.

BiP/Kar2p Purification from Total Cell Extracts of *S. cerevisiae*

Chromosomal integration of *Strep*-tag II – HDEL occurred at the C-terminus of *KAR2*, in parental strain BJ5464. Recombinant BiP was expressed in an overnight culture (15 mLs, 30°C, 275 rpm), and then inoculated into 500 mLs of YPD medium ($OD_{600} = 0.6$). Following approximately eight hours of growth at 30°C, mid-log phase was achieved ($OD_{600} = 1.8$) and cells were harvested. Cells were centrifuged at $3,000 \times g$ for 10 min at 4°C (Sorvall® RC6 PLUS, Thermo Electron Corporation) then resuspended in 50 mLs of lysis buffer containing 150 mM NaCl, 100 mM Tris, 1 mM EDTA, pH = 8.0, and complete, EDTA-free protease inhibitor cocktail tablets (Roche), per manufacturer's instructions. Cells were disrupted by homogenization at 23,000 psi (Model M-110L, Microfluidizer® Processor) and immediately centrifuged at $60,000 \times g$ and 4°C for 30 min (Beckman Coulter Optima™ Ultra centrifuge, 45 Ti rotor). Clear supernatant was placed in aliquots, frozen in liquid nitrogen, and stored at -80°C .

High-speed supernatant (4.6 mLs, equivalent to $\sim 80 OD_{600}$ of cells) was applied to a 2.5 mL column volume of *Strep*Tactin Superflow Agarose (Novagen) equilibrated in 150 mM NaCl, 100 mM Tris, 1 mM EDTA, 5 mM adenosine triphosphate (ATP), pH = 8.0. Overnight incubation of the resin occurred at 4°C with continuous rotation. Prior to purification, 5 mM of ATP (SIGMA-ALDRICH®) was added for 1 – 2 hrs. The resin was sequentially washed with 25 mLs of buffer (150 mM NaCl, 100 mM Tris, 1 mM EDTA, pH = 8.0). BiP-*Strep*-tag II - HDEL was eluted with 5 mLs of buffer containing 2.5 mM d-desthiobiotin (SIGMA-ALDRICH®). D-desthiobiotin is a derivative of biotin with decreased affinity; therefore, its use facilitates the regeneration of the column. Fractions of eluate were collected every 1.25 mLs. Representative fractions of this *Strep*-tag II/*Strep*Tactin purification were analyzed by SDS-PAGE and western blot analysis, as described in previous sections. All samples were normalized to volumes used throughout the purification protocol; thus, a mass balance was completed. The resultant recovery of BiP-*Strep*-tag II -HDEL was calculated as 54%. Regeneration of the column occurred per manufacturer's instructions. Reagents consisted of 150 mM NaCl, 100 mM Tris, 1 mM EDTA, and 1 mM hydroxyl-azophenyl-benzoic acid (HABA) at pH = 8.0. HABA was directly applied to the resin, thus ensuring the removal of D-desthiobiotin, as indicated by a color change (i.e. yellow to red).

Distribution of Plasmids

The full collection of plasmids and their sequences are available to non-commercial recipients at the non-profit plasmid repository Addgene (<http://www.addgene.org>).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Depiction of key components in pCY H/KDEL cassettes, adapted from Young and colleagues (77)

(A) Plasmid map of the template used for this cassette series, which contains C-terminal ER-retrieval sequences (e.g. denoted by a five-point star). (B) Generalized nucleotide sequence of all pCY H/KDEL cassettes, similar to modules derived from pFA6a-GFP(S65T) (22). Nucleotides corresponding to retrieval sequences, FEHDEL and MLKDEL, are identical to the final six amino acids of BiP/Kar2p and Scj1p, respectively, in *S. cerevisiae*. To amplify specific tags and selection markers, regions of primer homology are denoted in italics. Restriction sites have been identified, and corresponding nucleotides underlined. A polylinker was designed based on frequently-used codons of *S. cerevisiae* (38) as denoted by frequency (i.e. percentage of codon usage within the entire genome) and inserted between the *XmaI* and original *PacI* restriction site, thereby eliminating its uniqueness. The ADH1 terminator (T_{ADH1}) of *S. cerevisiae* is a component of all pCY H/KDEL plasmids that are derivatives of parental vectors consisting of *kanMX*. (C) Arrangement of all blue, green, yellow, and red fluorescent protein variants of the pCY H/KDEL series. (D) Epitope tags, *c*-myc and HA, contain codon-optimized (i.e. yeast-enhanced (yE)) sequences. Residues that maintain affinity for commercial antibodies are in bold. (E) A modified streptavidin tag, *Strep*-tag II, was constructed with an N-terminal thrombin cleavage site and C-terminal

HDEL retrieval sequence. The recognized cleavage site is denoted by (^). As a consequence of thrombin's enzymatic activity, remaining amino acids are underlined. The *Strep*-tag II epitope is shown as bold font. **(F)** General design for forward and reverse primers using standard homologous recombination techniques.

Figure 2.
(A) Expression of BiP/Kar2p fluorescent protein fusions implementing the HDEL retrieval sequence In-gel fluorescence analysis of GFP and DsRed variants fused to an ER resident protein, BiP, compared to parental strains. FP fusions were detected at excitation wavelengths of 457 nm, 488 nm, or 532 nm. **(B) Immunoblotting of the Hsp70 chaperone, BiP.** Western blot analysis shows corresponding lanes analogous to (A); protein bands were probed with anti-Kar2p and compared to total protein load via an appropriate control, anti-Actin. **(C) Comparison of full-length BJ5464 BiP-FP fusions via in-gel fluorescence analysis under non-reducing conditions.** FP fusions were detected at excitation wavelengths of 457 nm, 488 nm, or 532 nm. Oligomers and mixed disulfides were not detected by intrinsic fluorescence of the variants. **(D) Western blot analyses confirmed the formation of one species under non-reducing conditions.** Endogenous BiP (wildtype) was probed by anti-Kar2p and BiP-FP fusions by anti-GFP.

retrieval sequence (i.e. FEHDEL). Fluorescence (left) and DIC (right) images of the indicated strains acquired by confocal microscopy (Zeiss 780 confocal microscope, 100×/NA 1.46), as described in *Materials and Methods*. All scale bars are 5 μ m.

Figure 4.

(A) Expression of Pdi1p fluorescent protein fusions that incorporate the C-terminal HDEL retrieval sequence In-gel fluorescence analysis of GFP and DsRed variants fused to protein disulfide isomerase, Pdi1p, compared to parental strains. FP fusions were detected at excitation wavelengths of 457 nm, 488 nm, or 532 nm. **(B) Immunoblots detect fluorescent protein fusions.** Western blot analysis shows corresponding lanes analogous to (A); protein bands were probed with anti-GFP and compared to total protein via an appropriate loading control, anti-Tubulin. **(C) Live-cell imaging of Pdi1p fluorescent protein fusions localized to the ER.** Fluorescence (left) and DIC (right) images of the indicated strains acquired by confocal microscopy, as described in *Materials and Methods* (Zeiss 780 confocal microscope, 100 $\times$ /NA 1.46). **(D) Implementation and optimization of blue fluorescent protein variants, yECFP and yEmCFP, fused to Pdi1p.** Live-cell imaging of Pdi1p fusion proteins – fluorescence (left) and DIC (right) - were acquired with improved sensitivity provided by GaAsP detectors (Zeiss 780 confocal microscope, 100 $\times$ /NA 1.46) at an excitation of 458 nm. All scale bars are 5 μ m.

Figure 5.
(A) Expression of Scj1p GFP variants that implement the C-terminal KDEL retrieval sequence In-gel fluorescence analysis of yellow GFP variants fused to Scj1p compared to parental strains. GFP variants were excited at 488 nm. **(B) Immunoblot analysis of Scj1p fusion proteins.** Western blot analysis shows corresponding lanes; protein bands were probed with anti-GFP and compared to total protein load via an appropriate control, anti-Actin. **(C) Live-cell imaging of Scj1p GFP variants.** Fluorescence (left) and DIC (right) images of the indicated strains acquired by confocal microscopy (Zeiss 780 confocal microscope, 100 $\times$ /NA 1.46), as described in *Materials and Methods*. All scale bars are 5 μ m. **(D) Growth rates of *S. cerevisiae* strains constitutively expressing ER-resident proteins fused to Venus with selective H/KDEL retrieval sequences.** Time course experiments were performed for parental strain BJ5464 (filled circles, wildtype (WT)) and derivatives consisting of BiP/Kar2p (open triangles, CY2009), Pdi1p (open squares, CY2017), and Scj1p (open diamonds, CY2021) fused to Venus (e.g. pCY3051-01 or

pCY3052-01, respectively). Experiments were completed in triplicate and graphically represented as a semi-log plot. For each time point, the mean $\pm$ error (i.e. minimum or maximum value of the measured OD₆₀₀) is represented. To statistically evaluate the respective doubling times, two-sample t-tests were performed at the significance level of $\alpha = 0.5$. Results are shown in the inset of (D), where the mean doubling times, $\overline{t_d}$, are denoted by the vertical bars with error bars defined as the standard error of the mean. Statistical significance results in the corresponding p-values (numbers above vertical bars).

Figure 6. Recombinant BiP was successfully purified from *S. cerevisiae* under physiological conditions. (A) Expression of BiP-*Strep*-tag II, designed with an HDEL retrieval motif Western blot analysis of ER molecular chaperone BiP/Kar2p, expressed as a recombinant *Strep*-tag II fusion protein, compared to wildtype. Protein bands were probed with anti-*Strep*-tag® II. **(B) Illustration of *Strep*-tag II/*Strep*Tactin purification, schematic modified from Schmidt and Skerra (90).** Cell lysate containing host proteins and recombinant BiP is applied to immobilized *Strep*Tactin. Host proteins are removed by sequential washes. BiP-*Strep*-tag II - HDEL is then displaced via competition with a low concentration of D-desthiobiotin. To regenerate the column, the removal of D-desthiobiotin is accelerated by the addition of HABA. **(C) Purification strategy and recovery of BiP-*Strep*-tag II - HDEL.** Analysis of recombinantly expressed and purified BiP-*Strep*-tag II - HDEL. Initially, cleared cell lysate (Load, 4.6 mLs total) was applied to *Strep*Tactin resin (2.5 mLs column volume) and flow-through (FT, 4.6 mLs) was collected. The column was washed sequentially five times (W, 5 mLs each), followed by the elution of BiP-*Strep*-tag II - HDEL (E, 8 × 1.25 mLs with 2.5 mM D-desthiobiotin). All fractions were normalized to volumes used throughout the purification scheme.

S. cerevisiae genes containing ER-retrieval sequences

Table 1

Gene	Function ¹	ER-Retrieval Sequence ²	UPR Induction ³
BiP/KAR2	ER molecular chaperone that mediates protein folding and maturation, involved in protein import to the ER (i.e. translocation) and ER-associated degradation	FEHDEL	Yes
LHS1	Molecular Hsp70 chaperone of the ER, considered a nucleotide exchange factor for BiP/Kar2	ILHDEL	Yes
SCJ1	Hsp40 co-chaperone of the ER lumen involved in protein folding and maturation ER quality control lectin and is a member of the OS-9 protein family; integral subunit of the HRD ligase;	MLKDEL	Yes
YOS9	binds to glycans with terminal alpha-1,6 linked mannose on misfolded N-glycosylated proteins and participates in targeting proteins to ERAD	IEHDEL	ND
JEM1	ER co-chaperone that interacts with BiP in the ER lumen, required for nuclear membrane fusion during mating	KMNFG	Yes
SIL1	Nucleotide exchange factor of BiP/Kar2, required for ER translocation of nascent proteins	NERDEL	ND
PDH1	Protein disulfide isomerase, ER luminal resident protein essential for the formation of disulfide bonds	AIHDEL	Yes
ERO1	Thiol oxidase required for oxidative protein folding in the ER	YKLDIQ	Yes
EUG1	Protein disulfide isomerase of the ER lumen whose function overlaps with Pdi1	TVHDEL	Yes
MPD1	Member of PDI family of foldases; due to its interaction with Cne1p (calnexin), Mpd1p inhibits its chaperone activity	NKHDEL	ND
SED4	Integral ER membrane protein that stimulates Sar1p GTPase activity, involved in COPII vesicle budding	GLHDEL	ND
SEC20	Membrane glycoprotein V-SNARE involved in retrograde transport from the Golgi to ER, required for N- and O-glycosylation in the Golgi	VSHDEL	ND
FKB2	Membrane-bound peptidyl-prolyl cis-trans isomerase	DVKSAA	Yes

¹Modified from *Saccharomyces* Genome Database and referenced accordingly, <http://www.yeastgenome.org/>

²Bold defines specific ER-retrieval sequences, the final 6 residues of respective C-termini are provided to maintain consistency with FEHDEL/SEKDEL trafficking studies completed by Pelham and colleagues (16, 84)

³UPR induction in *S. cerevisiae* evaluated by the chemical treatment of cells (4)

Table 2

S. cerevisiae strains used in this study

Strain	Genotype	Primers	Source/[Reference]
B15464	MA7a. <i>ura3-52 tp1 leu2Δ his3Δ200 pep4::HIS3 prb1-Δ1.6R can1 GAL</i>	---	ATCC® 208288™ (74)
CY2007	MA7a. <i>ura3-52 tp1 leu2Δ his3Δ200 pep4::HIS3 prb1-Δ1.6R can1 GAL KAR2-Cerulean-HDEL::hphMX4</i>	HRF1 KAR2, HRR KAR2	This study
CY2008	MA7a. <i>ura3-52 tp1 leu2Δ his3Δ200 pep4::HIS3 prb1-Δ1.6R can1 GAL KAR2-yEpolylinker-yEmCFP-HDEL::hphMX4</i>	HRF2 KAR2, HRR KAR2	This study
CY2009	MA7a. <i>ura3-52 tp1 leu2Δ his3Δ200 pep4::HIS3 prb1-Δ1.6R can1 GAL KAR2-Venus-HDEL::kanMX</i>	HRF1 KAR2, HRR KAR2	This study
CY2010	MA7a. <i>ura3-52 tp1 leu2Δ his3Δ200 pep4::HIS3 prb1-Δ1.6R can1 GAL KAR2-yEpolylinker-yEVenus-HDEL::kanMX</i>	HRF2 KAR2, HRR KAR2	This study
CY2011	MA7a. <i>ura3-52 tp1 leu2Δ his3Δ200 pep4::HIS3 prb1-Δ1.6R can1 GAL KAR2-yEpolylinker-yECitrine-HDEL::kanMX</i>	HRF2 KAR2, HRR KAR2	This study
CY2012	MA7a. <i>ura3-52 tp1 leu2Δ his3Δ200 pep4::HIS3 prb1-Δ1.6R can1 GAL KAR2-yEpolylinker-yEmCitrine-HDEL::kanMX</i>	HRF2 KAR2, HRR KAR2	This study
CY2013	MA7a. <i>ura3-52 tp1 leu2Δ his3Δ200 pep4::HIS3 prb1-Δ1.6R can1 GAL KAR2-yEpolylinker-yEGFP-HDEL::kanMX</i>	HRF2 KAR2, HRR KAR2	This study
CY2014	MA7a. <i>ura3-52 tp1 leu2Δ his3Δ200 pep4::HIS3 prb1-Δ1.6R can1 GAL KAR2-mCherry-HDEL::hphMX4</i>	HRF1 KAR2, HRR KAR2	This study
CY2015	MA7a. <i>ura3-52 tp1 leu2Δ his3Δ200 pep4::HIS3 prb1-Δ1.6R can1 GAL PDI1-yEpolylinker-yECFP-HDEL::hphMX4</i>	HRF2 PDI1, HRR PDI1	This study
CY2016	MA7a. <i>ura3-52 tp1 leu2Δ his3Δ200 pep4::HIS3 prb1-Δ1.6R can1 GAL PDI1-yEpolylinker-yEmCFP-HDEL::hphMX4</i>	HRF2 PDI1, HRR PDI1	This study
CY2017	MA7a. <i>ura3-52 tp1 leu2Δ his3Δ200 pep4::HIS3 prb1-Δ1.6R can1 GAL PDI1-Venus-HDEL::kanMX</i>	HRF1 PDI1, HRR PDI1	This study
CY2018	MA7a. <i>ura3-52 tp1 leu2Δ his3Δ200 pep4::HIS3 prb1-Δ1.6R can1 GAL PDI1-yEpolylinker-yEVenus-HDEL::kanMX</i>	HRF2 PDI1, HRR PDI1	This study
CY2019	MA7a. <i>ura3-52 tp1 leu2Δ his3Δ200 pep4::HIS3 prb1-Δ1.6R can1 GAL PDI1-yEpolylinker-yEGFP-HDEL::kanMX</i>	HRF2 PDI1, HRR PDI1	This study
CY2020	MA7a. <i>ura3-52 tp1 leu2Δ his3Δ200 pep4::HIS3 prb1-Δ1.6R can1 GAL PDI1-mCherry-HDEL::hphMX4</i>	HRF1 PDI1, HRR PDI1	This study
CY2021	MA7a. <i>ura3-52 tp1 leu2Δ his3Δ200 pep4::HIS3 prb1-Δ1.6R can1 GAL SCJ1-Venus-KDEL::kanMX</i>	HRF1 SCJ1, HRR SCJ1	This study
W303	MA7a. <i>ade2-1 ura3-1 his3-11 tp1-1 leu2-3, 112 can1-100</i>	---	ATCC® 208353™ (76)
CY4001	MA7a. <i>ade2-1 ura3-1 his3-11 tp1-1 leu2-3, 112 can1-100 KAR2-mCherry-HDEL::hphMX4</i>	HRF1 KAR2, HRR KAR2	This study
CY4002	MA7a. <i>ade2-1 ura3-1 his3-11 tp1-1 leu2-3, 112 can1-100 SCJ1-Venus-KDEL::kanMX</i>	HRF1 SCJ1, HRR SCJ1	This study
BY4742	MA7a. <i>his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0</i>	---	EUROSCARF Y1000/ (75)
CY6006	MA7a. <i>his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 KAR2-yEpolylinker-Cerulean-HDEL::hphMX4</i>	HRF2 KAR2, HRR KAR2	This study
CY6007	MA7a. <i>his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 KAR2-yEpolylinker-yEGFP-HDEL::kanMX</i>	HRF2 KAR2, HRR KAR2	This study
CY6008	MA7a. <i>his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 KAR2-mCherry-HDEL::hphMX4</i>	HRF1 KAR2, HRR KAR2	This study
CY6009	MA7a. <i>his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 PDI1-yEpolylinker-yEGFP-HDEL::kanMX</i>	HRF2 PDI1, HRR PDI1	This study
CY6010	MA7a. <i>his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 PDI1-mCherry-HDEL::hphMX4</i>	HRF1 PDI1, HRR PDI1	This study
CY6011	MA7a. <i>his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 SCJ1-yEpolylinker-yEmCitrine-KDEL::kanMX</i>	HRF2 SCJ1, HRR SCJ1	This study

EUROSCARF (European *Saccharomyces cerevisiae* Archive for Functional Analysis)