JBC ACCELERATED COMMUNICATION

Overlapping function of Hrd1 and Ste24 in translocon quality control provides robust channel surveillance

Received for publication, September 25, 2020, and in revised form, October 6, 2020 Published, Papers in Press, October 8, 2020, DOI 10.1074/jbc.AC120.016191

Avery M. Runnebohm‡, Kyle A. Richards‡, Courtney Broshar Irelan, Samantha M. Turk, Halie E. Vitali, Christopher J. Indovina, and Eric M. Rubenstein*

From the Department of Biology, Ball State University, Muncie, Indiana, USA

Edited by George N. DeMartino

Translocation of proteins across biological membranes is essential for life. Proteins that clog the endoplasmic reticulum (ER) translocon prevent the movement of other proteins into the ER. Eukaryotes have multiple translocon quality control (TQC) mechanisms to detect and destroy proteins that persistently engage the translocon. TQC mechanisms have been defined using a limited panel of substrates that aberrantly occupy the channel. The extent of substrate overlap among TQC pathways is unknown. In this study, we found that two TQC enzymes, the ER-associated degradation ubiquitin ligase Hrd1 and zinc metalloprotease Ste24, promote degradation of characterized translocon-associated substrates of the other enzyme in *Saccharomyces cerevisiae*. Although both enzymes contribute to substrate turnover, our results suggest a prominent role for Hrd1 in TQC. Yeast lacking both Hrd1 and Ste24 exhibit a profound growth defect, consistent with overlapping function. Remarkably, two mutations that mildly perturb post-translational translocation and reduce the extent of aberrant translocon engagement by a model substrate diminish cellular dependence on TQC enzymes. Our data reveal previously unappreciated mechanistic complexity in TQC substrate detection and suggest that a robust translocon surveillance infrastructure maintains functional and efficient translocation machinery.

Approximately one-third of eukaryotic proteins enter the endoplasmic reticulum (ER) en route to their final destinations (1, 2). Many of these proteins use the conserved Sec61 translocon protein channel to cross the ER membrane (3). Translocation is essential to cellular viability (4, 5). Most eukaryotic polypeptides enter the ER via co-translational translocation (CTT). However, a substantial number of proteins cross the membrane following synthesis via post-translational translocation (PTT) (6). CTT and PTT utilize different translocon complexes. In *Saccharomyces cerevisiae*, CTT is mediated by a core heterotrimer composed of the Sec61 pore, Sbh1, and Sss1 (7). Proteins entering the ER via PTT utilize a heptamer composed of the core heterotrimer and an auxiliary tetramer containing Sec62, Sec63, Sec66, and Sec72 (8–10).

Proteins that aberrantly engage or persistently occupy (*i.e.* clog) the translocon obstruct the movement of other proteins into the ER (11, 12). The importance of protein translocation is underscored by the fact that cells have evolved at least three translocon quality control (TQC) mechanisms to detect and destroy such aberrant proteins (11–16). Conserved TQC mechanisms have been most intensively studied in yeast. ER-associated degradation (ERAD) of translocon-associated proteins (ERAD-T) is mediated by the ER-resident ubiquitin ligase Hrd1 (15). Mammalian apolipoprotein B (the major protein component of low-density lipoproteins) is targeted for degradation by the Hrd1 homolog gp78 if its translocation arrests (17). In ERAD of ribosome-associated proteins (ERAD-RA), the cytosolic ubiquitin ligase Ltn1/Rkr1 (listerin in mammals) promotes destruction of yeast proteins that simultaneously arrest in the ribosome and translocon (14). *In vitro* data indicate that ERAD-RA is conserved in mammalian systems (18). In a third TQC mechanism, the ER-localized zinc metalloprotease Ste24 (ZMPSTE24 in mammals) promotes the degradation of proteins engineered to clog the translocon, likely via substrate cleavage (12).

The extent of overlap of TQC pathways in substrate selection is not known. We investigated the functional redundancy between Hrd1 and Ste24. We found that two model TQC substrates are targeted for destruction, to different extents, by both Hrd1 and Ste24. Cells lacking both enzymes exhibit a synthetic growth defect, consistent with overlapping function. This phenotype is rescued by two mutations that preferentially impair PTT and reduce the extent of aberrant engagement by a model TQC substrate. We speculate that many naturally occurring TQC substrates engage the translocon post-translationally. Perturbations that selectively impair PTT are likely to reduce the frequency of clogging and, consequently, the cellular dependence on enzymes required for translocon unclogging. Our results indicate that TQC is a robust system characterized by functional redundancy of Hrd1- and Ste24-mediated protein degradation.

Results

Yeast lacking HRD1 and STE24 exhibit a synthetic growth phenotype

To investigate the relative contributions of Hrd1 and Ste24 to TQC, we generated yeast lacking genes encoding both

This article contains supporting information.

‡These authors contributed equally to this work.
* For correspondence: Eric M. Rubenstein, emrubenstein@bsu.edu.
Present Address for Avery M. Runnebohm: School of Medicine, Indiana University, Indianapolis, Indiana, USA.
Present Address for Courtney Broshar Irelan: Diabetes and Complications Therapeutic Area, Eli Lilly and Company, Indianapolis, Indiana, USA.
Present Address for Halie E. Vitali: Department of Investigative Toxicology, Eli Lilly and Company, Indianapolis, Indiana, USA.
Present Address for Christopher J. Indovina: Life Sciences Solutions, Thermo Fisher Scientific, Grand Island, New York, USA.

enzymes. We compared the growth of WT, *hrd1*Δ, *ste24*Δ, and *hrd1*Δ *ste24*Δ yeast. Yeast individually lacking either *HRD1* or *STE24* grew comparably to WT cells (Fig. 1*A*). However, *hrd1*Δ *ste24*Δ yeast exhibited a profound growth defect, consistent with Hrd1 and Ste24 possessing redundant function. Similarly, yeast lacking *STE24* and *UBC7* (which encodes the primary ubiquitin-conjugating enzyme that functions with Hrd1) grew more slowly than either *ubc7*Δ or *ste24*Δ yeast (Fig. 1*B*). We previously observed (and reproduce in Fig. 1*C*) that *hrd1*Δ *ltn1*Δ yeast grow similarly to WT yeast (14). We also generated yeast lacking *LTN1* and *STE24* and detected no genetic interaction. These results reveal a specific, negative genetic relationship between *HRD1* and *STE24*.

Hrd1 and Ste24 possess overlapping substrate specificity

Roles for Hrd1 and Ste24 in TQC have been inferred by their ability to target unique model substrates engineered to aberrantly engage the translocon. Hrd1 substrates are incompletely stabilized in *hrd1*Δ yeast, suggesting parallel degradative mechanisms (19). To query their specificity in TQC, we asked whether Hrd1 and Ste24 can target characterized substrates of the other enzyme.

The prototypical Hrd1 ERAD-T substrate is *Deg1**-Sec62 (Fig. 1*D*) (15). Following co-translational insertion of Sec62 transmembrane segments, the cytosolic N-terminal tail loops into the translocon via PTT (15). This engagement is stabilized by an adventitious disulfide bond between Sec62 and the interior of Sec61 (15, 20). Hrd1 targets *Deg1**-Sec62 for degradation following this aberrant translocation (15).

We investigated the contributions of Hrd1 and Ste24 to *Deg1**-Sec62 turnover. In WT cells, *Deg1**-Sec62 exhibited characteristic post-translational modification and rapid degradation (Fig. 1, *E* and *F*) (15). Loss of Hrd1 strongly but incompletely stabilized *Deg1**-Sec62, consistent with the existence of redundant targeting mechanisms. Cells lacking only Ste24 degraded *Deg1**-Sec62 with similar kinetics as WT cells. However, loss of *STE24* in the context of *HRD1* deletion modestly but significantly stabilized *Deg1**-Sec62 more than deletion of *HRD1* alone. This effect is partially obscured by the fact that *STE24* deletion reduced *Deg1**-Sec62 steady-state abundance (compare $t = 0$ for *hrd1*Δ and *hrd1*Δ *ste24*Δ). These results indicate that both Hrd1 and Ste24 contribute to *Deg1**-Sec62 degradation, with Hrd1 having the more prominent role.

We next determined whether Hrd1 promotes degradation of the model Ste24 substrate, Clogger (Fig. 1*D*). In Clogger, the ER-targeted soluble protein Pdi1 is fused to a rapidly folding version of dihydrofolate reductase (DHFR) (12). *N*-glycosylation sites are present upstream and downstream of the translocon-clogging DHFR moiety. The position of Clogger relative to the ER membrane (cytosolic, translocon-clogged, inserted) can be assessed based on differential migration by SDS-PAGE (Fig. 1*G*). Species with the lowest mobility are fully glycosylated and have been completely inserted into the ER lumen. Species with the greatest mobility are nonglycosylated cytosolic molecules that have not engaged the translocon. Intermediate species represent hemiglycosylated, translocon-clogging proteins. We collectively refer to the combined Clogger population that is clogged or cytosolic as "preinserted."

At steady state, the relative proportion of preinserted Clogger was significantly increased in both *ste24*Δ and *hrd1*Δ cells compared with WT yeast ($t = 0$; Fig. 1, *G* and *H*). This was also observed in cells harboring attenuated proteasomes (Fig. S1, *A* and *B*) (12). Further, loss of either Ste24 or Hrd1 partially but reproducibly stabilized total (Fig. 1*I*) and preinserted (Fig. 1*J*) Clogger populations. Loss of Ste24 had a greater impact on steady-state abundance of preinserted Clogger than *HRD1* deletion. By contrast, *HRD1* deletion had a stronger effect on turnover kinetics of both total and preinserted Clogger populations.

Loss of Ste24 and Hrd1 had subtly different effects on the steady-state distribution of Clogger isoforms. Cells lacking Ste24 preferentially enriched cytosolic Clogger (Fig. 1*G*; quantified in Fig. S1*C*). *ste24*Δ and *hrd1*Δ cells both enriched the clogged form relative to WT cells. Further, the clogged:cytosolic ratio at steady state was greater in *hrd1*Δ than in *ste24*Δ cells (Fig. S1*D*).

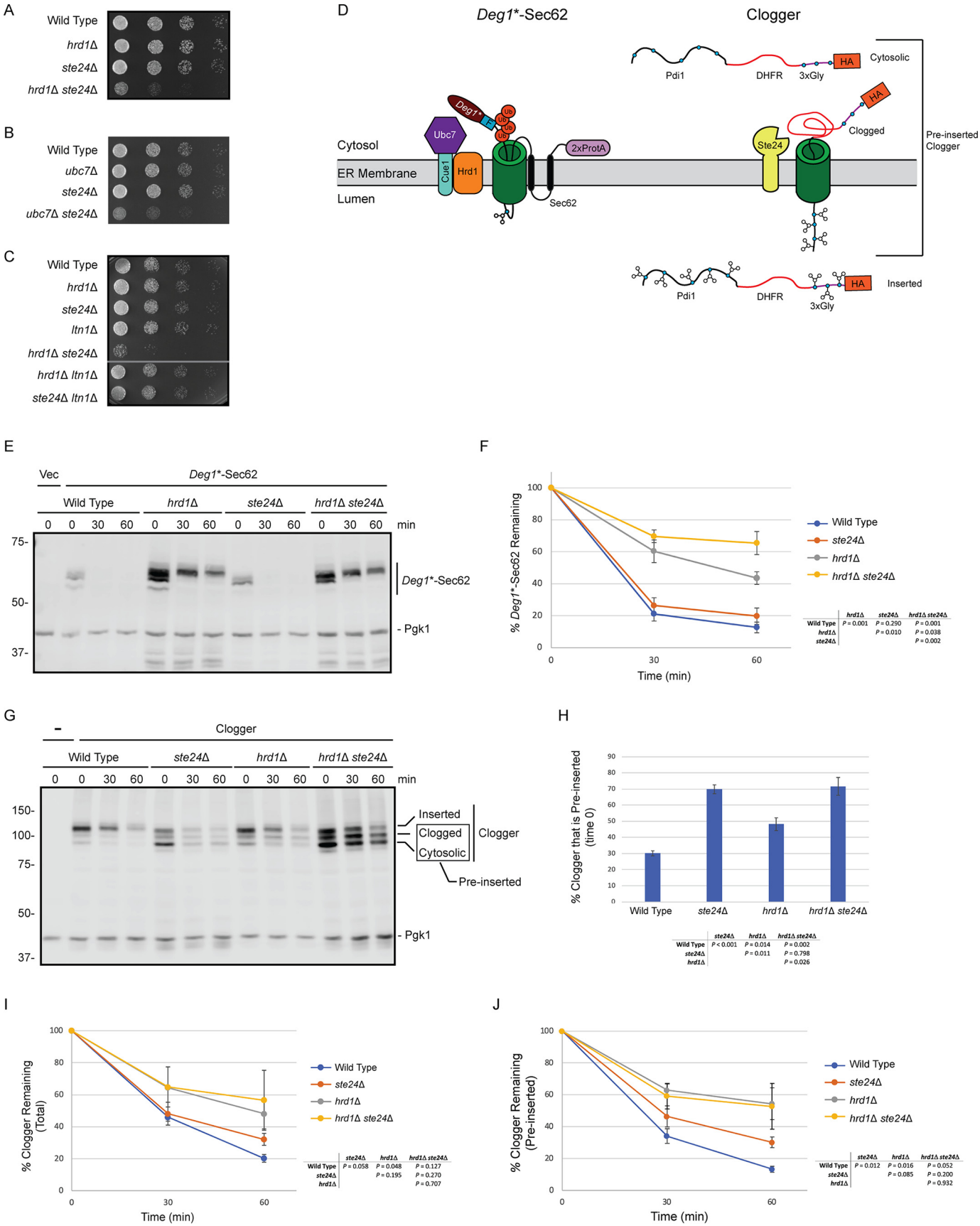
Simultaneous deletion of *STE24* and *HRD1* increased Clogger steady-state abundance relative to both single deletions (Fig. 1*G*). However, *hrd1*Δ *ste24*Δ cells did not additively or synergistically stabilize Clogger or increase the proportion of Clogger that was preinserted (Fig. 1, *I* and *J*). These observations indicate that Hrd1 and Ste24 both contribute to Clogger degradation, with Hrd1 having the more prominent role.

*C-terminal extension of Sec61 impairs post-translational translocation and rescues synthetic phenotype of hrd1*Δ *ste24*Δ *yeast*

We introduced a 13myc epitope tag to the C terminus of the essential Sec61 translocon pore subunit. This version of the translocon has been reported to be functional (21). Cells expressing sec61-13myc grew similarly to WT cells (Fig. 2*A*). Strikingly, this tag rescued the growth defect of *hrd1*Δ *ste24*Δ yeast.

We analyzed the extent of aberrant translocon engagement by *Deg1**-Sec62, which occurs via PTT (15), in cells expressing sec61-13myc. The N-terminal tail of the transmembrane protein *Deg1**-Sec62 becomes glycosylated after it enters the translocon. A 13myc epitope on Sec61 reduced the extent of *Deg1**-Sec62 glycosylation, consistent with reduced frequency of aberrant translocon engagement (Fig. 2, *B* and *E*).

We investigated the impact of the Sec61 C-terminal 13myc epitope on translocation of model post- and co-translationally translocated proteins. Carboxypeptidase Y (CPY) enters the ER via PTT. CPY can be engineered to undergo CTT by replacing its signal sequence with that of co-translationally imported protein Ost1; the hybrid protein is termed OPY (14, 22). Upon ER import, CPY and OPY become *N*-glycosylated and exhibit reduced electrophoretic mobility, as confirmed by endoglycosidase H (Endo H) sensitivity (Fig. 2*E*) (14, 22). CPY, but not OPY, translocation, was partially impaired in cells expressing sec61-13myc (Fig. 2, *C–E*). Thus, C-terminal tagging of Sec61 with 13myc preferentially impairs PTT.


A
Wild Type
hrd1Δ
ste24Δ
hrd1Δ ste24Δ
B
Wild Type
ubc7Δ
ste24Δ
ubc7Δ ste24Δ
C
Wild Type
hrd1Δ
ste24Δ
ltn1Δ
hrd1Δ ste24Δ
hrd1Δ ltn1Δ
ste24Δ ltn1Δ
D
Deg1*-Sec62
Clogger
Cytosolic
Pdi1
DHFR
3xGly
HA
Clogged
Pre-inserted Clogger
Inserted
Cytosol
ER Membrane
Lumen
Ubc7
Cue1
Hrd1
Ste24
2xProtA
Sec62
E
Vec
Deg1*-Sec62
Wild Type
hrd1Δ
ste24Δ
hrd1Δ ste24Δ
0 0 30 60 0 30 60 0 30 60 0 30 60 min
75-
50-
37-
Deg1*-Sec62
- Pgk1
F
% Deg1*-Sec62 Remaining
Time (min)
Wild Type
ste24Δ
hrd1Δ
hrd1Δ ste24Δ
G
-
Clogger
Wild Type
ste24Δ
hrd1Δ
hrd1Δ ste24Δ
0 0 30 60 0 30 60 0 30 60 0 30 60 min
150-
100-
75-
50-
37-
Inserted
Clogged
Cytosolic
Clogger
Pre-inserted
- Pgk1
H
% Clogger that is Pre-inserted (time 0)
Wild Type
ste24Δ
hrd1Δ
hrd1Δ ste24Δ
I
% Clogger Remaining (Total)
Time (min)
Wild Type
ste24Δ
hrd1Δ
hrd1Δ ste24Δ
J
% Clogger Remaining (Pre-inserted)
Time (min)
Wild Type
ste24Δ
hrd1Δ
hrd1Δ ste24Δ

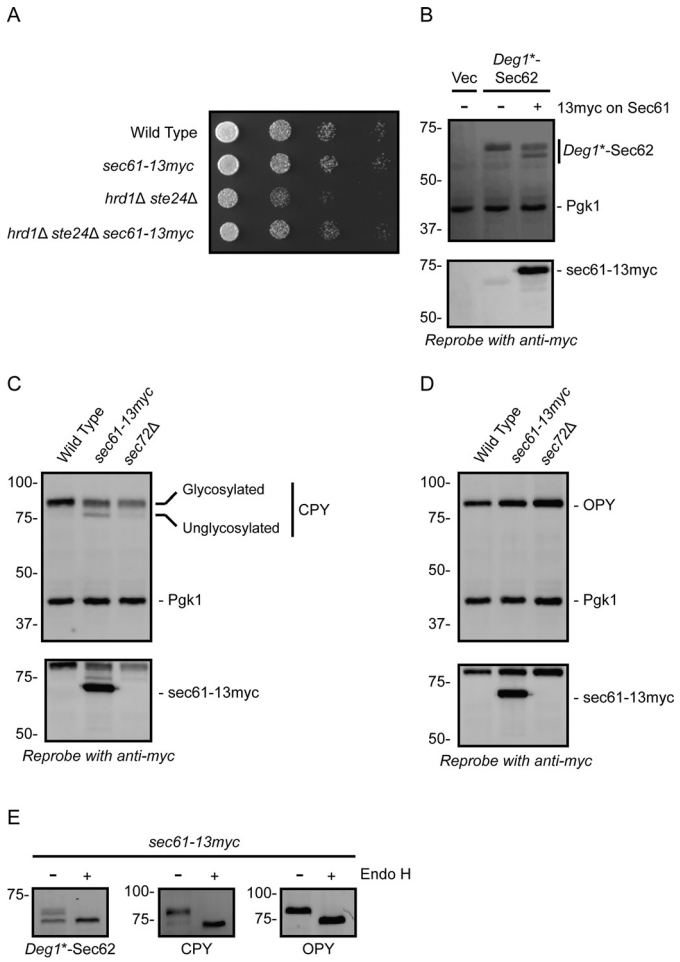


Figure 2. C-terminal extension of Sec61 rescues the synthetic phenotype of *hrd1Δ ste24Δ* yeast and impairs post-translational translocation. *A*, 6-fold serial dilutions of yeast of the indicated genotypes were spotted onto rich growth medium and incubated at 30°C. *B–D*, *top panels*, evaluation of steady-state abundance and migration of *Deg1**-Sec62, CPY, or OPY in WT yeast, yeast expressing sec61-13myc, or *sec72*Δ yeast (*C* and *D*). *Bottom panels*, membranes were reprobed with anti-myc antibodies to confirm sec61-13myc expression. *E*, lysates from cells expressing sec61-13myc and *Deg1**-Sec62, CPY, or OPY were incubated in the absence or presence of Endo H prior to Western blotting. Experiments in *A–D* were performed three times. The experiment in *E* was performed one time. *Vec*, empty vector.

Loss of SEC72 impairs post-translational translocation and rescues synthetic phenotype of hrd1Δ ste24Δ yeast

We hypothesized that phenotypic rescue of *hrd1*Δ *ste24*Δ yeast by sec61-13myc is due to impaired PTT. We determined whether deletion of *SEC72*, which encodes a translocon component required for efficient PTT (8, 23), also restores growth of *hrd1*Δ *ste24*Δ yeast. Individual deletion of *SEC72* had negligible impact on growth (Fig. 3*A*). However, like *sec61-13myc*, *SEC72* deletion rescued the *hrd1*Δ *ste24*Δ synthetic phenotype. Further, PTT of *Deg1**-Sec62 and CPY (but not CTT of OPY) was impaired in the absence of *SEC72* (Figs. 2, *C* and *D*, and 3, *B–D*), although not to the same extent as by Sec61 tagging.

Yeast lacking HRD1 and SEC66 exhibit a negative genetic interaction

Previous genome-wide analyses revealed a negative interaction between deletions of *STE24* and *SEC66* (24–27), which encodes a translocon component required for PTT (8, 28). We determined whether *hrd1*Δ *sec66*Δ yeast also exhibit a synthetic phenotype. We generated yeast expressing or lacking all combinations of *SEC66*, *HRD1*, and *DOA10* (an ER ubiquitin ligase with no characterized role in TQC (15)). Yeast lacking *SEC66* exhibited a modest growth defect compared with WT yeast (Fig. 4*A*), as previously documented (29, 30). Individual or simultaneous deletion of *HRD1* and *DOA10* did not impact cell fitness, consistent with earlier results (31). *hrd1*Δ *sec66*Δ yeast (but not *doa10*Δ *sec66*Δ yeast) exhibited a modest synthetic negative growth phenotype that was exacerbated at an elevated temperature (Fig. 4*B*). By contrast, we observed no genetic interaction between *HRD1* and *SEC72* (Fig. 4*C*). Phenotypic similarity of *hrd1*Δ *sec66*Δ and *ste24*Δ *sec66*Δ yeast supports a model in which Hrd1 and Ste24 possess related translocon-linked function.

Discussion

Protein translocation is essential for life. WT levels of fitness of cells lacking individual TQC enzymes likely reflect a robust translocon surveillance infrastructure enabled by functional redundancy. Indeed, we found simultaneous loss of Ste24 and Hrd1 (or its ubiquitin-conjugating enzyme Ubc7) dramatically attenuates cell fitness. Consistent with our results, negative genetic interactions between *UBC7* and *STE24* have been detected in multiple large-scale analyses (25, 27, 32, 33). Further, we observed that Hrd1 and Ste24 are capable, to different extents, of targeting model substrates of the other enzyme.

Hrd1 and Ste24 may not overlap in function with Ltn1. Simultaneous deletion of *LTN1* with either *HRD1* or *STE24* does not reduce fitness. Previous work demonstrated that Hrd1 does

Figure 1. Hrd1 and Ste24 have overlapping roles in translocon quality control. *A–C*, 6-fold serial dilutions of yeast of the indicated genotypes were spotted onto rich growth medium and incubated at 30°C. These experiments were performed three times. *D*, schematic representations of *Deg1**-Sec62 (*left*) and Clogger (*right*). *Deg1**-Sec62 consists of a modified form of the 67 N-terminal amino acids from yeast transcriptional repressor MATα2 (*Deg1**), a FLAG epitope (*blue rectangle* labeled *F*), the two-transmembrane protein Sec62, and two copies of protein A from *S. aureus*. Following insertion of the transmembrane segments of Sec62, the cytosolic N-terminal tail of *Deg1**-Sec62 loops into the translocon and becomes *N*-glycosylated. Hrd1 functions with Ubc7, anchored by Cue1, in targeting *Deg1**-Sec62 for ubiquitin-mediated (*red circles* labeled *Ub*) degradation. Clogger consists of the post-translationally translocated Pdi1, the rapidly folding DHFR, three engineered *N*-glycosylation sites (3xGly), and a hemagglutinin (*HA*) epitope. DHFR folding causes Clogger to clog the translocon, whereupon it is likely cleaved by the zinc metalloprotease Ste24. The position of Clogger relative to the ER membrane (cytosolic, translocon-clogged, or inserted) can be distinguished by extent of glycosylation. *Blue circles*, glycosylated amino acids. *E*, cycloheximide chase of *Deg1**-Sec62 in yeast of the indicated genotypes. *Deg1**-Sec62 (driven by the *MET25* promoter) was expressed from a yeast centromeric plasmid. *F*, percentage of *Deg1**-Sec62 remaining over time (normalized to Pgk1) in three trials of the experiment depicted in *E*. *G*, cycloheximide chase of Clogger in yeast of the indicated genotypes. Clogger (driven by the *GAL1* promoter) is chromosomally integrated at the *HO* locus. *H*, percentage of Clogger that is preinserted (clogged or cytosolic) at steady state ($t = 0$) in three trials of experiment depicted in *G*. *I*, percentage of total Clogger remaining over time (normalized to Pgk1) in three trials of experiment depicted in *G*. *J*, percentage of preinserted Clogger remaining over time (normalized to Pgk1) in three trials of experiment depicted in *G*. Two-tailed unpaired *t* tests were performed to compare the mean values of 60-min time points between curves in (*F*, *I*, and *J*) or proportions of Clogger that is preinserted at steady state (*H*). *P* values are indicated in tables *below* or to the *right* of graphs. *Error bars* depict standard error of mean. *Vec*, empty vector.

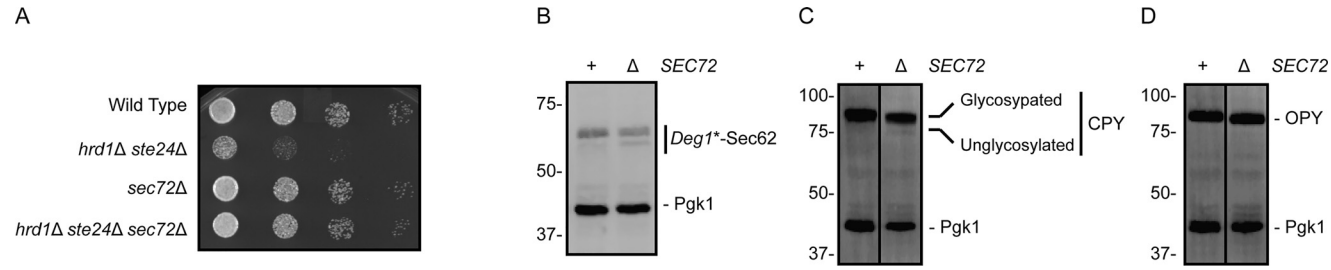


Figure 3. *SEC72* deletion rescues the synthetic phenotype of *hrd1Δ ste24Δ* yeast and impairs post-translational translocation. *A*, 6-fold serial dilutions of yeast of the indicated genotypes were spotted onto rich growth medium and incubated at 30°C. *B–D*, evaluation of steady-state abundance and migration of *Deg1**-Sec62, CPY, or OPY in WT and *sec72Δ* yeast. Experiments were performed three times.

not target model translationally and translocationally stalled Ltn1 substrates (14). This may reflect a fundamental difference in the TQC clientele of Ltn1 compared with that of Hrd1 and Ste24. Ltn1 function in TQC is likely a facet of its broader role in ribosome quality control (34, 35). Ribosome modification by the ubiquitin-like protein modifier UFM1 also promotes lysosomal degradation of some translationally stalled ER-targeted mammalian proteins (36). UFMylation is not conserved in fungi; other ribosome modifications may regulate quality control of translationally and translocationally stalled yeast proteins in parallel to Ltn1-mediated degradation.

In contrast to Ltn1 substrates, the best characterized TQC substrates of Hrd1 and Ste24 aberrantly engage the translocon post-translationally (12, 15). A substantial fraction of translocated yeast proteins are predicted to move into the ER via PTT (6). Numerous mammalian secretory proteins also likely use the PTT pathway (37, 38). We hypothesize that many naturally occurring translocon-clogging proteins are post-translationally translocated; these proteins may begin folding prior to channel engagement, as has been previously proposed (12). This is consistent with phenotypic rescue of *hrd1Δ ste24Δ* by mutations (*sec61-13myc* and *sec72Δ*) that specifically impair PTT. We show that these mutations reduce aberrant translocon engagement by *Deg1**-Sec62. Others have demonstrated that *SEC72* deletion reduces the frequency of aberrant translocon engagement by Clogger (12). When TQC mechanisms are compromised, unresolved translocon-clogging events are likely to accumulate, which is expected to impair normal ER import and cellular growth. Selective dampening of PTT (*i.e. sec61-13myc* or *sec72Δ*) likely reduces the rate of translocon clogging, even in the context of defective TQC, restoring growth to WT levels.

It is not clear how a 13myc epitope on Sec61 impairs PTT. A C-terminal appendage may interfere with translocon binding to PTT substrates or ER-targeting chaperones (39). Alternatively, 13myc might alter the structure or composition of the PTT complex.

*Deg1**-Sec62 degradation is largely independent of several Hrd1 co-factors that are required for turnover of Hrd1 substrates with luminal or intramembrane degradation signals (15). Whether Hrd1 co-factor independence is idiosyncratic of *Deg1**-Sec62 or whether these accessory proteins are broadly dispensable for Hrd1-dependent TQC remains to be determined. Interestingly, loss of the gene encoding derlin Dfm1, which contributes to degradation of some Hrd1 substrates, causes preinserted isoforms of Clogger to accumulate (12) and enhances toxicity of an engineered translocon-associated, oligomeric islet amyloid precursor protein expressed in yeast (40). In these contexts, Dfm1 has been proposed to recruit Cdc48 to extract Ste24-cleaved substrates from the translocon. Our discovery that Hrd1 contributes to Clogger degradation raises the possibility that Dfm1 functions as a Hrd1 co-factor in TQC. Future experiments will be performed to determine whether Hrd1 and its co-factors also modify toxicity or abundance of oligomeric islet amyloid precursor protein.

Although both Hrd1 and Ste24 contribute to destruction of two model aberrant translocon-associated proteins, the relative contributions of these enzymes in TQC are not equal. Hrd1 is the primary contributor to *Deg1**-Sec62 degradation; a role for Ste24 is only evident when Hrd1 is absent. *STE24* deletion has a greater impact on the relative steady-state abundance of preinserted Clogger, whereas *HRD1* deletion more strongly impairs degradation of these species. Together, these results suggest that Hrd1 plays a more prominent role in TQC substrate turnover than Ste24.

At steady state, the cytosolic form of Clogger accumulates most dramatically in *ste24Δ* yeast. By contrast, a greater proportion of preinserted protein is translocon-clogged in *hrd1Δ* cells. These observations suggest previously unappreciated mechanistic complexity in TQC substrate selection. Ste24 may preemptively cleave aberrant ER-targeted proteins prior to or in early stages of translocation, whereas Hrd1 may recognize proteins that have escaped Ste24 detection and subsequently clog the pore (see model in Fig. 4*D*). Early detection by Ste24 is supported by data indicating that Ste24 interacts with both clogged and cytosolic Clogger isoforms (12).

Lack of an additive stabilizing effect of *HRD1* and *STE24* deletion on preinserted Clogger is also consistent with a model in which Hrd1 and Ste24 function in a single degradative mechanism. However, disappearance of preinserted Clogger reflects a combination of degradation and conversion to the fully inserted species. The inserted isoform, which may arise via translocation prior to rapid folding of the DHFR domain or in a manner that requires unfolding of the DHFR translocon plug, is also cleared over time. Thus, the lack of increased stabilization in *hrd1Δ ste24Δ* cells may be due to the presence of additional parallel TQC mechanisms or the degradation of molecules that have reached the ER lumen. Further, a growth defect is only observed when both *HRD1* and *STE24* are knocked out, consistent with at least partially redundant function. Unambiguous assignment of distinct roles for Hrd1 and Ste24 in TQC and determination of

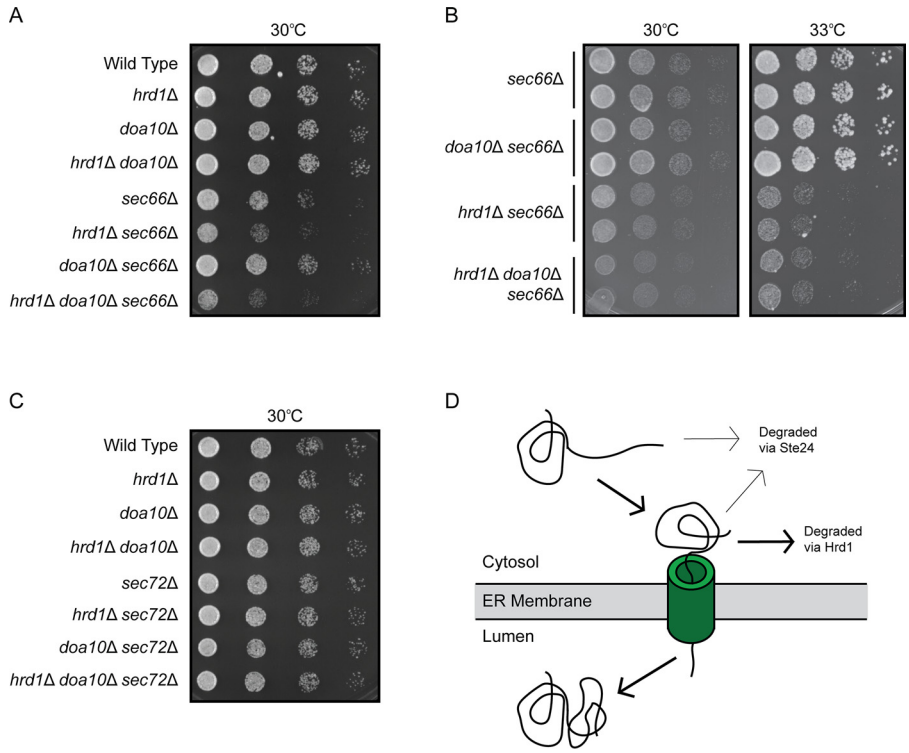


Figure 4. *hrd1Δ sec66Δ* yeast exhibit a growth defect. *A–C*, 6-fold serial dilutions of yeast of the indicated genotypes were spotted onto rich growth medium and incubated at the specified temperatures. The experiments presented in *A* and *C* were performed three times. The experiment presented in *B* was performed twice. *D*, hypothetical model depicting roles of Hrd1 and Ste24 in translocon quality control.

whether these enzymes function in a single mechanism or parallel pathways will be facilitated by discovery of substrates that more efficiently clog the translocon.

We recently observed both *Deg1**-Sec62 and Clogger are stabilized by ER stress (41). The extent to which substrate stabilization by ER stress reflects reduced function of Hrd1 or Ste24 is not known. In the previous study, we observed accumulation of the clogged isoform of Clogger (which we observed here to be preferentially enriched in *hrd1Δ* cells), consistent with inhibition of Hrd1 by ER stress. We speculate that impairment of TQC during ER stress is an adaptive response to stem protein import into an already overwhelmed ER.

Additional phenotypic similarities link Hrd1 and Ste24. Yeast lacking *HRD1* or *STE24* exhibit increased levels of ER stress (26) and sensitivity to ER stressors (42, 43). Combined loss of *HRD1* and *STE24* might be predicted to exacerbate ER stress induction and sensitivity, contributing to the synthetic phenotype of *hrd1Δ ste24Δ* yeast. Further, both *HRD1* and *STE24* exhibit a negative genetic relationship with *SEC66* (Refs. 25 and 32 and Fig. 4), which encodes a post-translational translocon subunit.

In the context of compromised TQC, deletion of *SEC66* and *SEC72* has opposite effects on cellular fitness. Given the central, complex role of the translocon in establishing the endomembrane system proteome, loss of different nonessential subunits may have differing impacts on cellular physiology. These may result from differences in translocation efficiency of a subset of proteins or alterations in the abundance or function of other translocon components.

Our genetic and biochemical data indicate that Hrd1 and Ste24 play important, overlapping roles at the translocon. Our results reveal novel complexity in TQC mediated by these enzymes. Subtle differences in efficiency of Hrd1 and Ste24 in targeting *Deg1**-Sec62 and Clogger suggest that these enzymes detect different features of translocon-clogging proteins or of clogged translocons themselves. Both Hrd1 and Ste24 interact with the translocon (12, 44, 45); Clogger expression stabilizes Ste24 association with the translocon (12). Further biochemical analyses will be necessary to understand the principles underlying TQC substrate recognition.

Experimental procedures

Yeast and plasmid methods

Yeast strains, plasmids, and primers used in this study are presented in Tables S1–S3, respectively. Construction of yeast strains and plasmids used in this study is described in the supporting information. Relevant genotypes were validated by PCR or, in the case of cells expressing sec61-13myc, anti-myc Western blotting (Fig. S2). Plasmids were introduced to yeast by lithium acetate transformation (46). Yeast were cultured in standard growth medium (46). For galactose induction of Clogger expression (47), yeast were grown overnight in synthetic-defined medium containing 4% galactose as the carbon source, diluted in fresh medium containing 4% galactose, and cultured to mid-exponential growth.

Cycloheximide chase, cell lysis, endoglycosidase H treatment, and Western blotting

Cycloheximide chase experiments (48), cell lysis, Western blotting (49, 50), and Endo H (New England Biolabs) treatment

(19) were performed as described. The following antibody dilutions were used: mouse anti-HA.11 (Clone 16B12; BioLegend) at 1:1,000 to detect Clogger; mouse anti–c-myc (Clone 9E10; BioLegend) at 1:1,000 to detect sec61-13myc; and mouse anti-phosphoglycerate kinase 1 (Pgk1; clone 22C5D8; Thermo Fisher Scientific) at 1:20,000. Primary antibodies were followed by incubation with Alexa Fluor 680–conjugated rabbit anti-mouse secondary antibody (Thermo Fisher Scientific) at 1:40,000. Alexa Fluor 680–conjugated rabbit anti-mouse secondary antibody was also used to directly detect the *Staphylococcus aureus* protein A epitope (51), present at the C termini of *Deg1**-Sec62, CPY, and OPY. Membranes were imaged using an Odyssey CLx IR imaging system and Image Studio Software (Li-Cor).

Data availability

All data are contained within the article.

Acknowledgments—We thank Douglas Bernstein, Paul Venturelli, Jason True, Phil Smaldino, Mark Hochstrasser, Christopher Hickey, Connor Bailey, and members of the Rubenstein, Bernstein, and Hochstrasser labs for insightful conversations. We thank Molly Dolan and Julia Niekamp for assistance in making and validating strains. We thank Seth Horowitz, Brian Voas, Kelsey Woodruff, Kamryn Kennedy, and Mahmoud Daraghmi for lab assistance. We thank Susan Calvin, Dee Hoffman, Melissa Schafstall, Pat Kleeberg, Janet Carmichael, Sarah Williams, Jackie Davis, Todd Davidson, Maria Bumbalough, Sarah Lee, and Chay Reigle for limitless support, patience, and kindness. We thank Robert Tomko, Mark Hochstrasser, and Maya Schuldiner for yeast strains. We thank Robert Tomko and Stefan Kreft for critical reading of this manuscript.

Author contributions—A. M. R., K. A. R., C. J. I., and E. M. R. conceptualization; A. M. R., K. A. R., C. B. I., S. M. T., H. E. V., C. J. I., and E. M. R. formal analysis; C. B. I. and E. M. R. supervision; A. M. R., K. A. R., C. B. I., and E. M. R. funding acquisition; A. M. R., K. A. R., C. B. I., S. M. T., H. E. V., C. J. I., and E. M. R. investigation; A. M. R. and E. M. R. methodology; E. M. R. writing-original draft; E. M. R. project administration; A. M. R., K. A. R., C. B. I., S. M. T., H. E. V., C. J. I., and E. M. R. writing-review and editing.

Funding and additional information—This work was supported by Ball State University ASPiRE Research Awards (to A. M. R., K. A. R., and C. B. I.), Indiana Academy of Science Senior Research Grants (to E. M. R. and K. A. R.), and National Institutes of Health Grant GM111713 (to E. M. R.). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Conflict of interest—The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations—The abbreviations used are: CPY, carboxypeptidase Y; CTT, co-translational translocation; DHFR, dihydrofolate reductase; Endo H, Endoglycosidase H; ER, endoplasmic reticulum; ERAD, ER-associated degradation; ERAD-T, ERAD of translocon-associated proteins; ERAD-RA, ERAD of ribosome-associated proteins; OPY, a variant of CPY engineered to possess the signal sequence from Ost1; PTT, post-translational translocation; TQC, translocon quality control.

References

1. Chen, Y., Zhang, Y., Yin, Y., Gao, G., Li, S., Jiang, Y., Gu, X., and Luo, J. (2004) SPD: a web-based secreted protein database. *Nucleic Acids Res.* **33,** D169–D173 CrossRef Medline
2. Choi, J., Park, J., Kim, D., Jung, K., Kang, S., and Lee, Y. H. (2010) Fungal secretome database: integrated platform for annotation of fungal secretomes. *BMC Genomics* **11,** 105 CrossRef Medline
3. Aviram, N., and Schuldiner, M. (2017) Targeting and translocation of proteins to the endoplasmic reticulum at a glance. *J. Cell Sci.* **130,** 4079–4085 CrossRef Medline
4. Stirling, C. J., Rothblatt, J., Hosobuchi, M., Deshaies, R., and Schekman, R. (1992) Protein translocation mutants defective in the insertion of integral membrane proteins into the endoplasmic reticulum. *Mol. Biol. Cell* **3,** 129–142 CrossRef Medline
5. Park, E., and Rapoport, T. A. (2012) Mechanisms of Sec61/SecY-mediated protein translocation across membranes. *Annu. Rev. Biophys.* **41,** 21–40 CrossRef Medline
6. Ast, T., Cohen, G., and Schuldiner, M. (2013) A network of cytosolic factors targets SRP-independent proteins to the endoplasmic reticulum. *Cell* **152,** 1134–1145 CrossRef Medline
7. Prinz, A., Hartmann, E., and Kalies, K. U. (2000) Sec61p is the main ribosome receptor in the endoplasmic reticulum of *Saccharomyces cerevisiae*. *Biol. Chem.* **381,** 1025–1029 CrossRef Medline
8. Harada, Y., Li, H., Wall, J. S., Li, H., and Lennarz, W. J. (2011) Structural studies and the assembly of the heptameric post-translational translocon complex. *J. Biol. Chem.* **286,** 2956–2965 CrossRef Medline
9. Wu, X., Cabanos, C., and Rapoport, T. A. (2019) Structure of the post-translational protein translocation machinery of the ER membrane. *Nature* **566,** 136–139 CrossRef Medline
10. Itskanov, S., and Park, E. (2019) Structure of the posttranslational Sec protein-translocation channel complex from yeast. *Science* **363,** 84–87 CrossRef Medline
11. Izawa, T., Tsuboi, T., Kuroha, K., Inada, T., Nishikawa, S., and Endo, T. (2012) Roles of dom34:hbs1 in nonstop protein clearance from translocators for normal organelle protein influx. *Cell Rep.* **2,** 447–453 CrossRef Medline
12. Ast, T., Michaelis, S., and Schuldiner, M. (2016) The protease Ste24 clears clogged translocons. *Cell* **164,** 103–114 CrossRef Medline
13. Arakawa, S., Yunoki, K., Izawa, T., Tamura, Y., Nishikawa, S., and Endo, T. (2016) Quality control of nonstop membrane proteins at the ER membrane and in the cytosol. *Sci. Rep.* **6,** 30795 CrossRef Medline
14. Crowder, J. J., Geigges, M., Gibson, R. T., Fults, E. S., Buchanan, B. W., Sachs, N., Schink, A., Kreft, S. G., and Rubenstein, E. M. (2015) Rkr1/Ltn1 ubiquitin ligase-mediated degradation of translationally stalled endoplasmic reticulum proteins. *J. Biol. Chem.* **290,** 18454–18466 CrossRef Medline
15. Rubenstein, E. M., Kreft, S. G., Greenblatt, W., Swanson, R., and Hochstrasser, M. (2012) Aberrant substrate engagement of the ER translocon triggers degradation by the Hrd1 ubiquitin ligase. *J. Cell Biol.* **197,** 761–773 CrossRef Medline
16. Voorhees, R. M., and Hegde, R. S. (2016) Toward a structural understanding of co-translational protein translocation. *Curr. Opin. Cell Biol.* **41,** 91–99 CrossRef Medline
17. Fisher, E. A., Khanna, N. A., and McLeod, R. S. (2011) Ubiquitination regulates the assembly of VLDL in HepG2 cells and is the committing step of the apoB-100 ERAD pathway. *J. Lipid Res.* **52,** 1170–1180 CrossRef Medline
18. von der Malsburg, K., Shao, S., and Hegde, R. S. (2015) The ribosome quality control pathway can access nascent polypeptides stalled at the Sec61 translocon. *Mol. Biol. Cell* **26,** 2168–2180 CrossRef Medline
19. Engle, S. M., Crowder, J. J., Watts, S. G., Indovina, C. J., Coffey, S. Z., and Rubenstein, E. M. (2017) Acetylation of N-terminus and two internal

amino acids is dispensable for degradation of a protein that aberrantly engages the endoplasmic reticulum translocon. *PeerJ* **5,** e3728 CrossRef Medline
20. Scott, D. C., and Schekman, R. (2008) Role of Sec61p in the ER-associated degradation of short-lived transmembrane proteins. *J. Cell Biol.* **181,** 1095–1105 CrossRef Medline
21. Carvalho, P., Stanley, A. M., and Rapoport, T. A. (2010) Retrotranslocation of a misfolded luminal ER protein by the ubiquitin-ligase Hrd1p. *Cell* **143,** 579–591 CrossRef Medline
22. Willer, M., Forte, G. M., and Stirling, C. J. (2008) Sec61p is required for ERAD-L: genetic dissection of the translocation and ERAD-L functions of Sec61P using novel derivatives of CPY. *J. Biol. Chem.* **283,** 33883–33888 CrossRef Medline
23. Lyman, S. K., and Schekman, R. (1997) Binding of secretory precursor polypeptides to a translocon subcomplex is regulated by BiP. *Cell* **88,** 85–96 CrossRef Medline
24. Schmidt, M., Hanna, J., Elsasser, S., and Finley, D. (2005) Proteasome-associated proteins: regulation of a proteolytic machine. *Biol. Chem.* **386,** 725–737 CrossRef Medline
25. Costanzo, M., Baryshnikova, A., Bellay, J., Kim, Y., Spear, E. D., Sevier, C. S., Ding, H., Koh, J. L., Toufighi, K., Mostafavi, S., Prinz, J., St Onge, R. P., VanderSluis, B., Makhnevych, T., Vizeacoumar, F. J., *et al.* (2010) The genetic landscape of a cell. *Science* **327,** 425–431 CrossRef Medline
26. Jonikas, M. C., Collins, S. R., Denic, V., Oh, E., Quan, E. M., Schmid, V., Weibezahn, J., Schwappach, B., Walter, P., Weissman, J. S., and Schuldiner, M. (2009) Comprehensive characterization of genes required for protein folding in the endoplasmic reticulum. *Science* **323,** 1693–1697 CrossRef Medline
27. Hoppins, S., Collins, S. R., Cassidy-Stone, A., Hummel, E., Devay, R. M., Lackner, L. L., Westermann, B., Schuldiner, M., Weissman, J. S., and Nunnari, J. (2011) A mitochondrial-focused genetic interaction map reveals a scaffold-like complex required for inner membrane organization in mitochondria. *J. Cell Biol.* **195,** 323–340 CrossRef Medline
28. Plath, K., Mothes, W., Wilkinson, B. M., Stirling, C. J., and Rapoport, T. A. (1998) Signal sequence recognition in posttranslational protein transport across the yeast ER membrane. *Cell* **94,** 795–807 CrossRef Medline
29. Deutschbauer, A. M., Williams, R. M., Chu, A. M., and Davis, R. W. (2002) Parallel phenotypic analysis of sporulation and postgermination growth in *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. U.S.A.* **99,** 15530–15535 CrossRef Medline
30. Marek, A., and Korona, R. (2013) Restricted pleiotropy facilitates mutational erosion of major life-history traits. *Evolution* **67,** 3077–3086 CrossRef Medline
31. Swanson, R., Locher, M., and Hochstrasser, M. (2001) A conserved ubiquitin ligase of the nuclear envelope/endoplasmic reticulum that functions in both ER-associated and Matα2 repressor degradation. *Genes Dev.* **15,** 2660–2674 CrossRef Medline
32. Costanzo, M., VanderSluis, B., Koch, E. N., Baryshnikova, A., Pons, C., Tan, G., Wang, W., Usaj, M., Hanchard, J., Lee, S. D., Pelechano, V., Styles, E. B., Billmann, M., van Leeuwen, J., van Dyk, N., *et al.* (2016) A global genetic interaction network maps a wiring diagram of cellular function. *Science* **353,** aaf1420 CrossRef Medline
33. Schuldiner, M., Collins, S. R., Thompson, N. J., Denic, V., Bhamidipati, A., Punna, T., Ihmels, J., Andrews, B., Boone, C., Greenblatt, J. F., Weissman, J. S., and Krogan, N. J. (2005) Exploration of the function and organization of the yeast early secretory pathway through an epistatic miniarray profile. *Cell* **123,** 507–519 CrossRef Medline
34. Bengtson, M. H., and Joazeiro, C. A. (2010) Role of a ribosome-associated E3 ubiquitin ligase in protein quality control. *Nature* **467,** 470–473 CrossRef Medline
35. Joazeiro, C. A. P. (2019) Mechanisms and functions of ribosome-associated protein quality control. *Nat. Rev. Mol. Cell Biol.* **20,** 368–383 CrossRef Medline
36. Wang, L., Xu, Y., Rogers, H., Saidi, L., Noguchi, C. T., Li, H., Yewdell, J. W., Guydosh, N. R., and Ye, Y. (2020) UFMylation of RPL26 links translocation-associated quality control to endoplasmic reticulum protein homeostasis. *Cell Res.* **30,** 5–20 CrossRef Medline
37. Lakkaraju, A. K., Thankappan, R., Mary, C., Garrison, J. L., Taunton, J., and Strub, K. (2012) Efficient secretion of small proteins in mammalian cells relies on Sec62-dependent posttranslational translocation. *Mol. Biol. Cell* **23,** 2712–2722 CrossRef Medline
38. Johnson, N., Powis, K., and High, S. (2013) Post-translational translocation into the endoplasmic reticulum. *Biochim. Biophys. Acta* **1833,** 2403–2409 CrossRef Medline
39. Tripathi, A., Mandon, E. C., Gilmore, R., and Rapoport, T. A. (2017) Two alternative binding mechanisms connect the protein translocation Sec71–Sec72 complex with heat shock proteins. *J. Biol. Chem.* **292,** 8007–8018 CrossRef Medline
40. Kayatekin, C., Amasino, A., Gaglia, G., Flannick, J., Bonner, J. M., Fanning, S., Narayan, P., Barrasa, M. I., Pincus, D., Landgraf, D., Nelson, J., Hesse, W. R., Costanzo, M., Consortium, A. T. D. G., Myers, C. L., *et al.* (2018) Translocon declogger Ste24 protects against IAPP oligomer-induced proteotoxicity. *Cell* **173,** 62–73.e9 CrossRef Medline
41. Buchanan, B. W., Mehrtash, A. B., Broshar, C. L., Runnebohm, A. M., Snow, B. J., Scanameo, L. N., Hochstrasser, M., and Rubenstein, E. M. (2019) Endoplasmic reticulum stress differentially inhibits endoplasmic reticulum and inner nuclear membrane protein quality control degradation pathways. *J. Biol. Chem.* **294,** 19814–19830 CrossRef Medline
42. Kapitzky, L., Beltrao, P., Berens, T. J., Gassner, N., Zhou, C., Wüster, A., Wu, J., Babu, M. M., Elledge, S. J., Toczyski, D., Lokey, R. S., and Krogan, N. J. (2010) Cross-species chemogenomic profiling reveals evolutionarily conserved drug mode of action. *Mol. Syst. Biol.* **6,** 451 CrossRef Medline
43. Parsons, A. B., Brost, R. L., Ding, H., Li, Z., Zhang, C., Sheikh, B., Brown, G. W., Kane, P. M., Hughes, T. R., and Boone, C. (2004) Integration of chemical-genetic and genetic interaction data links bioactive compounds to cellular target pathways. *Nat. Biotechnol.* **22,** 62–69 CrossRef Medline
44. Kadowaki, H., Satrimafitrah, P., Takami, Y., and Nishitoh, H. (2018) Molecular mechanism of ER stress-induced pre-emptive quality control involving association of the translocon, Derlin-1, and HRD1. *Sci. Rep.* **8,** 7317 CrossRef Medline
45. Schäfer, A., and Wolf, D. H. (2009) Sec61p is part of the endoplasmic reticulum-associated degradation machinery. *EMBO J.* **28,** 2874–2884 CrossRef Medline
46. Guthrie, C., and Fink, G. R. (2004) *Guide to Yeast Genetics and Molecular and Cell Biology*, pp. 1–933, Elsevier, San Diego, CA
47. Avci, D., and Lemberg, M. K. (2015) Clipping or extracting: two ways to membrane protein degradation. *Trends Cell Biol.* **25,** 611–622 CrossRef Medline
48. Buchanan, B. W., Lloyd, M. E., Engle, S. M., and Rubenstein, E. M. (2016) Cycloheximide chase analysis of protein degradation in *Saccharomyces cerevisiae*. *J. Vis. Exp.* **110,** e53975 Medline
49. Kushnirov, V. V. (2000) Rapid and reliable protein extraction from yeast. *Yeast* **16,** 857–860 CrossRef Medline
50. Watts, S. G., Crowder, J. J., Coffey, S. Z., and Rubenstein, E. M. (2015) Growth-based determination and biochemical confirmation of genetic requirements for protein degradation in *Saccharomyces cerevisiae*. *J. Vis. Exp.* **96,** e52428 Medline
51. Hjelm, H., Hjelm, K., and Sjöquist, J. (1972) Protein A from *Staphylococcus aureus*: its isolation by affinity chromatography and its use as an immunosorbent for isolation of immunoglobulins. *FEBS Lett.* **28,** 73–76 CrossRef Medline