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

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Abstract

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.

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...
enzymes. We compared the growth of WT, hrd1Δ, ste24Δ, and hrd1Δ ste24Δ yeast. Yeast individually lacking either HRD1 or STE24 grew comparably to WT cells (Fig. 1A). However, hrd1Δ ste24Δ 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 ubc7Δ or ste24Δ yeast (Fig. 1B). We previously observed (and reproduce in Fig. 1C) that hrd1Δ ln1Δ 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 hrd1Δ 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. 1D) (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 hrd1Δ and hrd1Δ ste24Δ). 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. 1D). 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. 1G). 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 ste24Δ and hrd1Δ 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. 1I) and preinserted (Fig. 1J) 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. 1G; quantified in Fig. S1C). ste24Δ and hrd1Δ cells both enriched the clogged form relative to WT cells. Further, the clogged/cytosolic ratio at steady state was greater in hrd1Δ than in ste24Δ cells (Fig. S1D).

Simultaneous deletion of STE24 and HRD1 increased Clogger steady-state abundance relative to both single deletions (Fig. 1G). However, hrd1Δ ste24Δ 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 hrd1Δ ste24Δ 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. 2A). Strikingly, this tag rescued the growth defect of hrd1Δ ste24Δ 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. 2E) (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.
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Figure 1. Hrd1 and Ste24 have overlapping roles in transloco 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. 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 sec72A 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 hrd1Δ ste24Δ 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 hrd1Δ ste24Δ yeast. Individual deletion of SEC72 had negligible impact on growth (Fig. 3A). However, like sec61-13sec61-13myc, SEC72 deletion rescued the hrd1Δ ste24Δ synthetic phenotype. Further, PTT of Deg1*-Sec62 and CPY (but not CNOT 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 hrd1Δ sec66Δ 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. 4A), as previously documented (29, 30). Individual or simultaneous deletion of HRD1 and DOA10 did not impact cell fitness, consistent with earlier results (31). hrd1Δ sec66Δ yeast (but not doa10Δ sec66Δ yeast) exhibited a modest synthetic negative growth phenotype that was exacerbated at an elevated temperature (Fig. 4B). By contrast, we observed no genetic interaction between HRD1 and SEC72 (Fig. 4C). Phenotypic similarity of hrd1Δ sec66Δ and ste24Δ sec66Δ 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). Furthermore, 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 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 sec72A 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.

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consistent with phenotypic rescue of engagement, as has been previously proposed (12). This is concluded by Clogger (12). When TQC mechanisms are compromised, the lack of an additive stabilizing effect of HRD1 and STE24 deletion on preinserted Clogger is also consistent with a 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. 4D). 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 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 induced 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.

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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 hrd1D 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
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(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 1:20,000. 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 Degl−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.

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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.

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