Defining human ERAD networks through an integrative mapping strategy

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Proteins that fail to correctly fold or assemble into oligomeric complexes in the endoplasmic reticulum (ER) are degraded by a ubiquitin- and proteasome-dependent process known as ER-associated degradation (ERAD). Although many individual components of the ERAD system have been identified, how these proteins are organized into a functional network that coordinates recognition, ubiquitylation and dislocation of substrates across the ER membrane is not well understood. We have investigated the functional organization of the mammalian ERAD system using a systems-level strategy that integrates proteomics, functional genomics and the transcriptional response to ER stress. This analysis supports an adaptive organization for the mammalian ERAD machinery and reveals a number of metazoan-specific genes not previously linked to ERAD.

Approximately one-third of the eukaryotic proteome consists of secreted and integral membrane proteins that are synthesized and inserted into the ER, where they must correctly fold and assemble to reach functional maturity¹. ER quality control refers to the processes simultaneously monitoring deployment of correctly folded proteins and assembled complexes to distal compartments, while diverting folding-incompetent, mutant or unassembled polypeptides for proteasomal degradation through the process of ERAD (reviewed in refs 2–4). An ever-growing list of sporadic and genetic human disorders have been associated with ER quality control, illustrating the pivotal role these processes play in governance of protein trafficking⁵.

Many of the individual components thought to underlie ERAD have been identified through genetic and biochemical analyses in Saccharomyces cerevisiae and mammals⁶,⁷ and point towards a mechanism mediated by a network of topologically and compartmentally restricted, partially redundant protein complexes⁷,⁸,⁹. ERAD is a vectorial process whereby coordination of ERAD components across three subcellular compartments (ER lumen, lipid bilayer and cytoplasm) must occur to effectively distinguish, target and deliver misfolded substrates for degradation. Exclusion of the ubiquitin–proteasome system (UPS) from the ER lumen necessitates that substrates traverse the ER membrane to be degraded, but the molecular identity and mechanism of the required dislocation apparatus remains controversial⁹,¹⁰,¹¹.

Ubiquitin E3 ligases play central functional and organizational roles in ERAD (ref. 9). In yeast, the E3s Hrd1 and Doa10, which contain cytoplasmically oriented RING domains that recruit distinct ubiquitin-conjugating enzymes and form functional complexes by scaffolding shared ERAD-related factors,¹²–¹⁶, seem to be sufficient to degrade all ERAD substrates¹⁶,¹⁷. ERAD substrates with luminal or membrane folding lesions utilize Hrd1 (refs 16,18), whereas those with cytoplasmic lesions rely on Doa10 (refs 16,17,19). In contrast to yeast, at least ten different E3s have been implicated in mammalian ERAD (ref. 20), possibly reflecting an evolutionary adaptation to the broader substrate range imposed by the more complex metazoan proteome. Three mammalian E3s, gp78, Hrd1 and TEB4, share similar domain and topological organization, but scant sequence homology, with their yeast orthologues Hrd1 (orthologue of gp78 and Hrd1) and Doa10 (orthologue of TEB4). Uncovering how the organization of E3-containing membrane complexes allows them to access substrates in the ER lumen/membrane and recruit the cytoplasmic dislocation/extraction apparatus is crucial to establishing a comprehensive understanding of ERAD.

Here, we have employed a systematic, multilayered approach that integrates high-content proteomics, functional genomics and gene expression to elucidate the interconnectivity and organization of ERAD in mammals (Supplementary Fig. S1). These studies have allowed us to generate an integrated physical and functional map of the ERAD system in the mammalian ER.

RESULTS
Mapping the mammalian ERAD interaction network
We employed a high-content proteomics strategy to map the mammalian ERAD interaction network, starting with 15 S-tagged baits consisting of proteins previously identified as ERAD pathway...
components in biochemical studies or by orthology to components identified in yeast (Supplementary Table S1, Primary). After confirming ER localization in HeLa cells (Supplementary Fig. S2) and stable expression of each full-length S-tagged bait in HEK293 cells (data not shown), protein complexes captured by S-protein affinity purification from detergent-solubilized lysates were analysed by liquid chromatography–tandem mass spectrometry (LC–MS/MS). Interactions were initially assessed for all baits by independently analysing pulldowns from cells lysed in digitonin (Supplementary Table S2) or the more stringent detergent Triton X-100 (Supplementary Table S3). Total spectral counts for each captured protein were subsequently evaluated with the Comparative Proteomics Analysis Software Suite21,22 (CompPASS; Supplementary Methods). CompPASS employs a database of interacting proteins (including data from baits in this study and 102 unrelated proteins described previously21) and comparative metrics to determine the likelihood of validity of interactors. The CompPASS parameter WDN-score22, which integrates the abundance, uniqueness and reproducibility of an interacting protein, was used to identify high-confidence candidate interacting proteins (HCIPs) for the ERAD network. Previous studies demonstrated that >68% of identified HCIPs were validated in subsequent biochemical analyses23, a rate of validation that is well above other high-throughput approaches to study protein–protein interactions23. In our study, interacting proteins surpassing a stringent threshold score of WDN > 1.0 were designated as HCIPs (Supplementary Tables S2a and S3a). Interacting proteins scoring below this cutoff may still represent bona fide interactions (full list in Supplementary Tables S2b and S3b).

In addition to revealing interconnections among primary baits, this analysis uncovered 10 HCIPs that had no previous relationship with ERAD. Seven (FAM8A1, UBAC2, KIAA0090, TTC35, C15orf24, TMEM111 and COX4NB) are functionally uncharacterized open reading frames and two (E-Syt1 and MMGT1, also known as TMEM32) are implicated in cellular processes unrelated to quality control. The HCIP TXD16 (also known as ERp90) was recently suggested to be involved in ERAD (ref. 24). On the basis of their identification as HCIPs with multiple ERAD components in both digitonin and Triton X-100, high spectral counts and predicted ER localization (criteria described in Methods), these 10 HCIPs were introduced into the proteomic workflow to iteratively expand and validate the network (Supplementary Fig. S1, Secondary). Three proteins previously implicated in mammalian ERAD (TEB4, RNF5 and HERP) could not be sufficiently expressed and were omitted. Ultimately, our ERAD network analysis included 25 baits, of which 9 seem to be unique to metazoans. No correlation was observed between bait abundance and the total number of interactions and HCIPs identified (Supplementary Fig. S3a). From 3,325 individual proteins identified by MASCOT in digitonin and 2,971 in Triton X-100 (Supplementary Tables S2b and S3b), CompPASS identified 320 and 202 HCIPs, respectively, for the 25 baits (Supplementary Tables S2a and S3a). These HCIPs correspond to 143 and 97 non-redundant proteins with 71 HCIPs of interest, previously uncharacterized for a role in ERAD (see Methods for selection criteria, Supplementary Table S4 and Fig. S3b). Over 50% of HCIPs are ER/membrane localized, and gene ontology analysis indicates diverse functionality with significant over-representation in folding, ubiquitin and catabolic processes (Supplementary Fig. S3c,d).

Overview of the mammalian ERAD interaction network

Unbiased hierarchical clustering of all HCIPs identified for each bait in both detergents was used to assemble interaction data into a coherent network (Fig. 1). Four of the digitonin clusters define subnetworks organized around the E3s Hrd1 (clusters 1D and 6D) and gp78 (clusters 3D and 8D), indicating a central role in organization of the mammalian ERAD system. Both Hrd1 and gp78 clustered with established integral membrane, luminal and cytoplasmic ERAD components (clusters 1D and 8D) as well as, separately, with most 26S proteasome subunits (clusters 3D and 6D). Cluster 2D defines a macromolecular complex of previously uncharacterized proteins that we have designated the mammalian ER membrane complex (mEMC, discussed below) to reflect its orthology to a complex associated with the unfolded protein response (UPR) in yeast25. Cluster 4D confirms the previously reported interactions of the AAA+ ATPase VCP (also known as p97) with an integral membrane binding partner VIMP (refs 26,27) and cytosolic NGly1 (ref. 28), while revealing interactions of VIMP and UBXD2 with the VCP accessory protein UBE4A, a Ufd2 orthologue implicated in ubiquitin chain extension29. In addition to confirming the ERFAD–SEL1L interaction30, cluster 5D validated the recently reported interaction of ERFAD with TXD16 (ref. 24), reinforcing the connection between ERAD and oxidative protein folding/ unfolding. Cluster 7D contains several HCIPs in complex with Derlin-1 and Derlin-2, including the Ca2+-sensing protein extended-synaptotagmin 1 (E-Syt1; ref. 31), the Ras superfamily member ARL6IP and YIF1B, both implicated in protein trafficking32,33.

Of the eight prominent clusters identified in digitonin-solubilized cells, only cluster 2 remained intact with Triton X-100 lysis. Four clusters (1, 3, 5 and 8) were fragmented into discrete subclusters, and three (4, 6 and 7) were fully disrupted. On the basis of these clusters (Fig. 1), we merged individual interactomes (Supplementary Fig. S4) to construct a topologically rendered, detailed interaction map of the mammalian ERAD network in digitonin and Triton X-100 (Fig. 2). The interaction network for ERAD (INfERAD) was arranged around clusters identified for Hrd1–SEL1L, gp78 and the mEMC subnetworks, with those components located centrally reflecting shared interactions between clusters.

As with any systems-based analysis of interaction networks, our analysis was not exhaustive, and therefore we sought to integrate data from public protein–protein interaction resources (STRING). However, as ERAD components are poorly represented in this database (Supplementary Table S5) and other online resources (BIOGRID and MINT), interactions identified in this study were instead mapped together with pairwise interactions reported previously (Supplementary Table S5 and Fig. S5). These combined data sets contain over 250 interactions, reflecting the organizational complexity of the mammalian ERAD system.

The Hrd1–SEL1L subnetwork

Our proteomic analysis confirmed the E3 Hrd1 and its established cofactor SEL1L as a prominent nexus for ERAD. Nearly all previously reported interactions of the Hrd1–SEL1L complex (Supplementary Table S5) were validated by our data set, which also uncovered
several unreported interactors including FAM8A1; LONP2, a putative Lon-protease (with OS-9); CPVL, a putative carboxypeptidase (with XTP3-B); the stress-inducible haem oxygenase HMOX1 (also known as HO1); and two components of the sterol biosynthetic pathway, HMG-CoA reductase (HMGCR) and squalene synthetase (FDFT1). The rate-limiting enzyme in cholesterol synthesis, HMGCR, is subject to strict feedback regulation whereby sterol end products induce its degradation by ERAD (refs 12,34,35). Although evidence supports a role for gp78 in the degradation of HMGCR in mammalian cells16, the Hrd1 pathway degrades HMGCR in both yeast (Hmg2; ref. 12) and Drosophila27. Identification of HMGCR as a Hrd1 HCIP lends strength to the possibility that Hrd1 also plays a role in HMGCR degradation in mammals39.

The integrity of the Hrd1–SEL1L subnetwork was strongly influenced by solubilization conditions. All Hrd1 HCIPs except FAM8A1 (Fig. 1a, cluster 1Ta) were lost in Triton X-100, whereas the complexes containing SEL1L, OS-9 and other luminal components were preserved (Fig. 1a, cluster 1Tb), consistent with all upstream (luminal) interactions being mediated through SEL1L, which is linked to Hrd1 by a Triton X-100-labile association39.

The gp78 subnetwork

Cluster analysis exposed a reciprocally co-precipitating complex consisting of the E3 gp78, an uncharacterized UBA domain-containing polytopic protein UBAC2, the membrane-embedded, VCP-binding protein UBXD8, and Derlin-1 and Derlin-2. The high degree of interconnectivity indicates that gp78 and its cognate E2 (UBE2G2) and UBAC2 comprise a transmembrane pathway for ERAD that shares essential cytoplasmic (for example, VCP and 26S proteasomes) and integral membrane components (UBXD8 and Derlin-2) with the Hrd1–SEL1L cluster. The recently described protein TMUB1 (ref. 40) was found in the gp78 cluster, as was BRI3BP, hitherto unlinked to ER. Signal peptide peptidase (SPP, also known as HM13) and a number of poorly characterized integral membrane proteins (TMEM201, TMEM43, LRRC59 and CLPTM1) were also linked through UBAC2. In contrast to the Hrd1–SEL1L cluster, most HCIPs associated with the gp78 subnetwork were stable in both detergents (Supplementary Table S2, S3 and S6). Disruption of the Hrd1–SEL1L cluster in Triton X-100 caused the shared cytoplasmic interactors VCP and UBE2G2 to cluster with gp78, probably reflecting their direct binding to the carboxy-terminal cytoplasmic domain of gp78 (refs 41,42). These data

Figure 1 Hierarchical cluster analysis of CompPASS-identified high-confidence candidate interaction proteins (HCIPs). Hierarchical clustering of HCIPs for interactions present in digitonin (left) and Triton X-100 (right). Prominent HCIP clusters identified in digitonin (1–8D) and Triton X-100 (1–5 and 8T) were manually selected and are highlighted below. The colour of the square indicates the WD-score.

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ERAD E3s are associated with the 26S proteasome

Strikingly, nearly all subunits of the 26S proteasome were captured with Hrd1 and gp78 in digitonin lysates. Although not all of these interactions reached our stringent criteria to qualify as HCIPs (19/32 for gp78; 15/31 for Hrd1; Supplementary Table S6), the fact that gp78 captured all subunits of the 20S core particle and most subunits of the 19S regulatory particle indicates an intimate, perhaps direct interaction of gp78 with the 26S proteasome. Proteasome components, indicates an intimate, perhaps direct interaction of the ERAD E3s and 26S proteasomes (Supplementary Fig. S6). Persistence of these interactions in Triton X-100, together with the observation that proteasome subunits X-100, together with the observation that proteasome subunits were not identified as HCIPs of other ERAD interaction network with Hrd1 and gp78 in digitonin lysates. Although not all of these interactions reached our stringent criteria to qualify as HCIPs (19/32 for gp78; 15/31 for Hrd1; Supplementary Table S6), the fact that gp78 captured all subunits of the 20S core particle and most subunits of the 19S regulatory particle indicates a significant connection of the ERAD E3s and 26S proteasomes (Supplementary Table S6 and Fig. S6). Persistence of these interactions in Triton X-100, together with the observation that proteasome subunits were not identified as HCIPs of other ERAD interaction network components, indicates an intimate, perhaps direct interaction of gp78 (and possibly Hrd1) with the 26S proteasome. Proteasome stability requires ATP and without it, dissociation into 20S core and 19S regulatory particles can occur. The excess of 20S core particle subunits over 19S regulatory subunits captured with gp78 and Hrd1 (Supplementary Fig. S6) raises the possibility that E3–proteasome connections may be linked independently of the 19S regulatory particle, perhaps through direct interactions with 20S or through alternative adaptors.

The gp78 HCIP PSME4 (also known as PA200) was identified in a screen for 26S proteasome activators and originally reported as a nuclear protein with a possible role in DNA repair. PA200 can assemble with 20S and 19S subunits to form hybrid 26S proteasomes and a crystal structure of the apparent yeast orthologue Blm10 indicates that it interacts directly with proteasome α-subunits. The functional significance of the PA200 interaction is unclear, but its presence in gp78 (but not Hrd1) complexes indicates that there may be heterogeneous 26S proteasome populations associated with the ER membrane and ERAD.

The mEMC subnetwork

The detergent-stable mEMC (Fig. 1, cluster 2) was initially identified through KIAA0090, an uncharacterized, putative type I integral membrane glycoprotein detected as a Derlin-1/2 HCIP (Fig. 1 and Supplementary Table S2). With KIAA0090 as bait, we identified five additional HCIPs (TTC35, MMGT1, C15orf24 and COX4NB), which reciprocally co-precipitated each other, and four additional proteins (TMEM111, C19orf63, C14orf122 and TMEM93; Supplementary Fig. S4). The mEMC comprises ten unique subunits, whereas its yeast counterpart seems to contain six (Fig. 2). Although the function of the mEMC is unknown, three subunits (KIAA0090, TMEM111 and TTC35) were identified as HCIPs of UBAC2 and Derlin-2, indicating a close link between this complex and ERAD components implicated in ubiquitin recognition and protein dislocation.

### Table of Determined Constituents of the Mammalian ER Membrane Complex

| Protein Name | Length (aa) | Locale | SC | SGD ID |
|--------------|-------------|--------|----|--------|
| KIAA0090    | 262         | IMP    | Emc1 | YL045C |
| MMGT1       | 131         | IMP    | Emc5 | YL027C |
| TMEM85      | 183         | IMP    | Emc4 | YGL231C|
| TMEM111     | 260         | IMP    | Emc3 | YKL207W|
| TTC35       | 297         | Cyto.  | Emc2 | YAR088C|
| TMEM93      | 110         | IMP    | Emc6 | YLL014W|
| C15orf24    | 242         | IMP    | -   | -      |
| C14orf122   | 208         | Cyto.  | -   | -      |
| COX4NB      | 210         | Cyto.  | -   | -      |
| C19orf63    | 262         | IMP    | -   | -      |

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shRNA-mediated refinement of ERAD E3 ligase subnetworks.

(a-q) S-tagged ERAD baits were transiently co-expressed with the indicated shRNAs in HEK293 cells. All complexes were affinity purified (AP) in 1% digitonin and analysed by immunoblotting.

S-prot, S-protein; FF, firefly. (a) XTP3-B–S expression, probe for Hrd1 and SEL1L simultaneously. (b) Co-expression of myc-UBE2J1 and XTP3-B–S expression, probe for FAM8A1 and SEL1L. (c) XTP3-B–S expression, probe for Hrd1 and SEL1L simultaneously. (d) Incorporation of refinements (a–d) to the S-prot complex. (e) Co-expression of myc–UBE2G2 and S–SEL1L, probe for myc and SEL1L. (f) Co-expression of myc–UBE2J1 and Hrd1–S, probe for myc and SEL1L. (g) Co-expression of myc–UBE2J1 and AUP1–S, probe for myc and SEL1L. (h) Co-expression of myc–UBE2J1 and S–SEL1L, probe for myc and Hrd1. (i) AUP1–S expression, probe for Hrd1 and SEL1L. (j) AUP1–S expression, probe for gp78 and SEL1L. (k) Refined interaction map for Hrd1 complex. (l) UBAC2–S expression with UBXD8 knockdown, probe for gp78. (m) UBAC2–S expression with gp78 knockdown, probe for UBXD8. (n) UBAC2–S expression with gp78 knockdown, probe for Derlin-2. (o) UBAC2–S expression with gp78 knockdown, probe for gp78. (p) UBAC2–S expression with Derlin-2 knockdown, probe for gp78. (q) Refined interaction map for the gp78 complex. Uncropped images of blots are shown in Supplementary Fig. S12.
Deconvolving the ERAD interaction network with RNA interference

To begin to decipher the organization within the mammalian ERAD interaction network, we systematically analysed the Hrd1–SEL1L and gp78 subnetworks. Co-expression of S-tagged proteins with short hairpin RNAs (shRNAs) targeting central subnetwork nodes was used to ascertain the requirement of each component to maintain individual interactions (Fig. 3). Following SEL1L knockdown, XTP3-B interactions with Hrd1 (Fig. 3a), UBE2J1 (Fig. 3b) and FAM8A1 (Fig. 3c) were abolished, and OS-9 lost its connection to Hrd1 (Fig. 3d). LC–MS/MS analyses confirmed that XTP3-B and OS-9 affinity-purified complexes from cells lacking SEL1L lost their interactions with all downstream membrane and cytosolic components (for example Hrd1, data not shown). These data verify the essential role that SEL1L plays in scaffolding luminal, substrate-recognition elements to the Hrd1 transmembrane complex30,39,48,49, and reveal the independent interactions of XTP3-B and OS-9 with the Hrd1–SEL1L node (Fig. 3e).

Hrd1–SEL1L subnetwork connections to integral membrane and cytosolic ERAD components differed in that SEL1L knockdown did not affect the Hrd1–FAM8A1 (Fig. 3f) or Hrd1–UBE2J1 interactions (Fig. 3g). Similarly, loss of Hrd1 failed to sever the connections between SEL1L–UBE2J1 (Fig. 3h), SEL1L–AUP1 (Fig. 3i) or AUP1–UBE2G2 (Fig. 3j). Thus, both Hrd1 and SEL1L bind to UBE2J1, either directly or through a factor not identified in our proteomic analysis. Hrd1 knockdown abolished the SEL1L–FAM8A1 interaction (Fig. 3f), indicating that SEL1L and FAM8A1 independently bind to Hrd1. This conclusion is reinforced by the maintenance of the Hrd1–FAM8A1 interaction in Triton X–100 where SEL1L is lost (Fig. 1). These data refine the molecular topology of the Hrd1–SEL1L complex, and identify FAM8A1 as an obligate, SEL1L-independent partner of Hrd1 (Fig. 3k).

A second prominent, highly interconnected subnetwork is composed of gp78, Derlin-2, UBAC2 and UBXD8 (Fig. 2). Knockdown of UBXD8 did not disrupt the gp78–UBAC2 interaction (Fig. 3i), nor did knockdown of gp78 affect UBXD8–UBAC2 (Fig. 3m,n). Although gp78 binding was lost, maintenance of the UBXD8–UBAC2 interaction in Triton X–100 indicates that their organization occurs independently of gp78 (Fig. 1). However, the UBXD8–gp78 interaction was abrogated by knockdown of UBAC2 (Fig. 3o) but not Derlin-2 (Fig. 3p). These data allow refinement of the gp78 subnetwork topology (Fig. 3q) and identify a role for UBAC2 in the recruitment of UBXD8 to the gp78 complex.

Functional genomic analysis of ERAD components

To assess their functional roles in substrate degradation, we monitored the effect of RNA interference (RNAi)-mediated knockdown of individual ERAD components on steady-state fluorescence levels of fluorescent ERAD substrate reporters21,30–37. Cell lines stably expressing GFP fusions representing three major topological classes of ERAD substrates: luminal-glycosylated (null Hong Kong variant of α1-antitrypsin (A1ATNHK), luminal-non-glycosylated (A1ATNSEH–QQO and mutant transhydrogen TTRD186C) and integral membrane-glycosylated (CFTRΔP508; Fig. 4a) substrates were employed. We also included the AMPA-type glutamate receptor subunit GluR1, as it is retained in the ER and degraded in a UPS-dependent manner (Supplementary Fig. S7). Cell lines expressing the cytosolic proteasome substrate GFPα (ref. 54) and GFP served as controls for ERAD-independent effects that might alter UPS function, reporter gene expression or GFP fluorescence intensity.

All substrate reporter lines responded to proteasome inhibition with time-dependent increases in mean GFP fluorescence (Fig. 4b). Expression of a dominant-negative VCP mutant (H317A; ref. 52) severely impaired degradation of only the ERAD substrates, but not GFPα (Fig. 4c), in agreement with the strong dependence of ERAD pathways on VCP and 26S proteasomes. The mannosidase inhibitor kifunensine selectively inhibited the degradation of A1ATNHK (Fig. 4c), consistent with an established requirement for mannose trimming of this glycoprotein for ERAD (refs 55,56). GluR1 and CFTRΔP508 are also glycoproteins (Supplementary Fig. S7c), but were unaffected by kifunensine (Fig. 4c), indicating that mannose trimming is unlikely to be the dominant signal committing them to degradation, or that there is redundant, glycan-independent targeting for these polytopic proteins. These data reflect an implicit requirement for multiple, substrate-specific recognition elements within the ERAD interaction network to deliver substrates to shared degradation machinery.

To identify the individual factors required for substrate degradation, we generated an shRNA library targeting genes implicated in ERAD (Fig. 4d and Supplementary Table S5) and monitored their impact on the mean GFP fluorescence of reporter cell lines (Fig. 4e and Supplementary Table S7). Any shRNA that significantly stabilized an ERAD reporter was selected for further validation by re-screening through all other reporter lines and confirmation of knockdown (see Methods and Supplementary Fig. S8). Each substrate seemed to rely on a unique set of individual ERAD components for degradation (Supplementary Table S7), which is illustrated as a hierarchically clustered heat map for comparison (Fig. 4f). Of the 59 components our library targeted, only the non-ATPase subunit of the 19S regulatory particle PSMD2 and VCP were essential for all ERAD substrates (Fig. 4f). GFPα was stabilized by knockdown of PSMD2, but not VCP, mimicking the effects of MG132 and VCPΔH317A (Fig. 4b,c) and validating the strategy of using shRNA-mediated gene silencing with ERAD reporters to interrogate the contribution of individual components to the overall degradation process. Hierarchical cluster analysis demonstrated that substrates were segregated by topology (luminal versus integral membrane), but not by glycosylation (Fig. 4f). Moreover, a surprising degree of heterogeneity within each substrate’s requirement profile was observed, especially for substrates utilizing the same central ERAD components (for example Hrd1, discussed below). These characteristic patterns indicate that the ERAD system operates largely as an adaptive network, in which unique combinations of common components process individual substrates. Such an adaptive mechanism could be explained by the formation of substrate-specific subcomplexes or by a multisubunit complex that utilizes discrete sets of components to achieve substrate-specific degradation.

An adaptive mechanism for Hrd1-dependent degradation

We merged the heat map of shRNA-mediated impairment for each substrate (Fig. 4f) with the comprehensive ERAD interaction network (Supplementary Fig. S5) to generate integrated substrate-specific snapshots of the physical and functional networks responsible for degradation (Supplementary Figs S9–S11). Loss of either E3 in the network impacted degradation in a substrate-specific manner. Hrd1 knockdown stabilized topologically disparate substrates including A1ATNSEH, A1ATNSE–QQO, TTRD186C and GluR1, but had little effect on GFPα or...
Figure 4 Functional genomic screen to identify essential substrate-specific ERAD components. (a) Localization and topology of GFP reporters (TTRD18G–GFP, A1ATNHK–GFP, A1ATNHK–GQG–GFP, GFP–GluR1, GFP–CFTR<sup>F508</sup> and GFP<sup>Δ</sup>F508) and GFP<sup>Δ</sup>F508. (b) Time course of relative mean GFP fluorescence intensity levels for each ERAD reporter cell line treated with MG132 (10 μM). Cyto., cytosolic. (c) Heat maps reflecting the normalized fold change in mean GFP fluorescence intensity of ERAD reporter lines transfected with wild-type or dominant-negative VCP (wild-type or H317A, top panel) and time course of treatment with kifunensine (30 μM, bottom panel). Fold change in mean GFP fluorescence intensity was normalized to the levels measured for each reporter at the 3 h time point of MG132 treatment, and thus a degradation score of 3 is equivalent to the impairment induced by 3 h MG132 treatment. (d) Target composition of the shRNA library. (e) Overview of the functional genomic screen. (f) Hierarchically clustered heat map of the normalized fold change in mean GFP fluorescence intensity of ERAD reporter lines transfected with 30 shRNA constructs (30 μM, bottom panel). A re-screen of MTAP<sup>Δ</sup>F508 (Fig. 4f,g). Instead, CFTR<sup>Δ</sup>F508 was stabilized following gp78 knockdown, as previously reported<sup>77</sup>. Substrates utilizing Hrd1 did not share a common dependence on Hrd1–SEL1L subnetwork components. Whereas FAM8A1 and SEL1L were essential for degradation of
Figure 5  Coordinated ER stress response of ERAD genes. qRTPCR results for validated and suspected ERAD components following treatment of HEK293 cells with tunicamycin (10 μg ml⁻¹, 6 h). Data are presented as fold induction (log₂) normalized to β-actin. Tunicamycin-induced expression changes in ERAD genes plotted as groups according to: (a) fold induction of gene expression represented by functional category, and (b) fold induction of gene expression from a mapped onto the ERAD interactome from Fig. 2. Additional genes of interest are presented alongside the induction map.

Coordinate regulation of ERAD genes by the unfolded protein response

Expression of more than half of the ERAD genes in our network, including the Hrd1–SEL1L subnetwork (Fig. 5) and other known UPR targets (for example, BiP and HERP), was induced by tunicamycin (Fig. 5). In contrast, gp78 and other ER-resident
E3s responded only weakly (Fig. 5). All but one of the mEMC components were transcriptionally upregulated by tunicamycin (Fig. 5); in yeast, only EMC3 is upregulated by the UPR (refs 25, 58).

Figure 6 Characterization of the Hrd1-binding partner FAM8A1.
(a) Domain structure and interaction network of FAM8A1, aa, amino acids. (b) Immunoprecipitation (IP) with anti-FAM8A1 from HEK293 digitonin-soluble lysates was analysed by immunoblotting with the indicated antibodies. (c) Consensus TOPCONS prediction of FAM8A1 membrane orientation (http://topcons.cbr.su.se). The reliability index indicates the likelihood for consensus prediction at each position using a sliding 21 amino-acid window. Cyto., cytosolic; TMD, transmembrane domain. (d) HEK293 membrane fractions incubated with 1 M NaCl, 0.1 M Na2CO3 at pH 12 or 1% SDS. Following 100,000 g centrifugation, equal volumes of soluble (S) and pellet (P) fractions were analysed by western blotting with anti-FAM8A1. (e) HeLa cells expressing S–FAM8A1 or Hrd1–S were permeabilized with digitonin or Triton X-100 to allow antibody access to cytosolic epitopes or cytosolic and luminal epitopes, respectively, immunostained and analysed by fluorescence microscopy. Scale bar, 10 μm. (f) Hrd1–S-expressing HEK293 cell lysates separated on a continuous 10–40% sucrose gradient. S-tagged Hrd1 protein complexes were affinity purified from each 1 ml fraction (fractions 1–12) or from 150 mg whole-cell lysate (10% AP), and analysed by western blotting for Hrd1 (S-tag), SEL1L and FAM8A1. (g) Heat map representing the normalized change in mean GFP fluorescence intensity (20,000 cells, n = 3) of the indicated ERAD reporter cell lines following transfection with the indicated Hrd1, SEL1L and FAM8A1 plasmids. GFP indicates dead fluorescent protein, a non-fluorescent GFP variant. Data are represented as a normalized heat map as in Fig. 4c. Uncropped images of blots are shown in Supplementary Fig. S12.

ERAD components identified within the Hrd1–SEL1L and gp78 subnetworks
FAM8A1 was identified as a previously uncharacterized component of the Hrd1–SEL1L subnetwork (Fig. 6a). Immunoprecipitation of endogenous FAM8A1 captured Hrd1 and SEL1L (Fig. 6b), confirming that FAM8A1 is a bona fide interactor of both components. Resistance to extraction from purified microsomes by high salt concentration or pH conditions support predictions for FAM8A1 as an integral membrane protein with three membrane-spanning domains (TOPCONS, Fig. 6c,d), and limited proteolysis of FAM8A1-containing microsomes (data not shown) and immunodetection of an aminoterminal epitope tag in semipermeabilized cells (Fig. 6e) established the cytosolic localization of the N terminus. A complex isolated with S-tagged Hrd1 contained both FAM8A1 and SEL1L, confirming FAM8A1 as a component of this E3 ligase complex (Fig. 6f).

Disrupting the stoichiometry of the Hrd1 E3 complex by FAM8A1 knockdown (Fig. 3f) or wild-type Hrd1 overexpression (Fig. 6g) impaired degradation of TTRD18G while enhancing that of GluR1. TTRD18G degradation was restored or enhanced when Hrd1 was co-expressed with SEL1L (Fig. 6g). Similarly, FAM8A1 overexpression (or its RDD domain, amino acids 230–413) impaired TTRD18G but not GluR1 degradation, whereas a cytoplasmic N-terminal fragment (FAM8A11–229) affected neither (Fig. 6g). The dominant-negative effect of FAM8A1D18G on TTRD18G stability implies that Hrd1 interacts with FAM8A1 through its RDD domain and that its cytoplasmic N-terminal region is required for Hrd1-mediated degradation of luminal substrates. Collectively, our results establish FAM8A1 as a binding partner and potential regulator of Hrd1-dependent ERAD.

UBAC2, identified as a UBXD8 interaction partner (Fig. 7a), is predicted to be a rhomboid family pseudoprotease similar to the Derlin proteins9 that also contains a putative C-terminal UBA domain. A native interaction between the two was validated by endogenous co-precipitation (Fig. 7b). Functionally, UBAC2 knockdown stabilized the Hrd1 substrate TTRD18G–GFP (Fig. 7c), indicating potential coordination between the two ubiquitin ligase complexes. Both the UBAC2 C terminus (amino acids 304–344) and the N terminus of UBXD8 (2–52) show a high degree of conservation with residues essential for ubiquitin binding in UBA domains (Fig. 7d), and their predicted cytosolic localization positions them appropriately for ubiquitin binding (Fig. 7e). Whereas a recombinant UBAC2 C-terminal fragment (amino acids 293–344) was sufficient to capture polyubiquitin chains from HEK293 cell lysates at a level comparable...
**DISCUSSION**

The application of high-content proteomics to identify interconnectivity within defined functional networks has been used with success to map high-resolution interaction landscapes for several complex mammalian protein networks. In this study, we have integrated the mammalian ERAD interaction landscape with gene expression strategies to uncover new components. Our analysis integrates interaction and functional data from the present study into a framework consisting of six functional modules that execute the principal ERAD

**Figure 7** Characterization of UBAC2, a ubiquitin-binding ERAD component. (a) Predicted domain structure and interaction network of UBAC2. (b) Immunoprecipitation (IP) with anti-UBXD8 from HEK293 digitonin-soluble lysates was analysed by western blotting with the indicated antibodies. (c) Analysis of multiple UBAC2-targeting shRNAs on the Hrd1 substrate TTR\(^{128}\)–GFP by flow cytometry. FF, firefly. (d) Sequence alignment of the predicted UBA domains from UBAC2 (304–344) and UBXD8 (8–53) with characterized human and yeast UBA domains. (e) HeLa cells expressing C-terminally S-tagged UBAC2 or gp78 were permeabilized, immunostained and analysed by fluorescence microscopy as in Fig. 6e. Scale bar, 10 \(\mu\)m. (f) Recombinantly expressed UBA domains of hPlic2, UBXD8 and UBAC2 were coupled to Affi-Gel and incubated with HEK293 cell lysates (\(\pm 10\mu\)M MG132, 6 h). Samples were separated by SDS–PAGE, and ubiquitin binding was determined by immunoblotting with anti-ubiquitin. Uncropped images of blots are shown in Supplementary Fig. S12.
Figure 8 Functional integration of mammalian ERAD networks. The schematic model of the ERAD protein interaction network is topologically organized with respect to the ER membrane and arranged as an array of six colour-coded functional modules. Individual components from this study (baits or HCIPs) are indicated as nodes with reported interactions (black) and previously unknown components (red). Similarly, reported interactions confirmed in this study (black lines) and previously unknown interactions (red lines) are shown. Symbols for protein–protein interactions, UPR induction and functional requirements are indicated in the legend. Inter-module interactions represent terminate either at the specific node within a module that establishes the link with the module periphery or at the module itself (where there are interactions with multiple components and that module is a single complex; for example, the mEMC or proteasome). Asterisks indicate components that were identified by proteomics, but exhibited a subthreshold CompPASS score (WDP-score < 1.0).

activities: substrate recognition, dislocation, extraction, ubiquitylation and degradation (proteasome), as well as the EMC whose function is unknown at present. Submodules are grouped together on the basis of predicted structural and topological features, and on an unbiased analysis of network interconnectivity of the proteins represented in each group. The ERAD system can thus be viewed as a distributed network, organized around central ubiquitin ligase modules for Hrd1 and gp78 that cooperate with components of the membrane-embedded dislocation and the cytoplasmically-oriented substrate extraction modules. These interconnections are likely to ensure secure coupling between substrate dislocation/extraction and ubiquitin conjugation. The Hrd1 and gp78 complexes contain submodule-specific factors and share interactions with ERLIN1/2 and UBE2G2. The Hrd1 submodule has four main connections to other modules. Three are mediated through SEL1L, which connects Hrd1 to the upstream luminal substrate recognition machinery, as well as to the downstream dislocation module through Derlin-2 and the substrate extraction module through UBXD8. Direct interactions between the last two proteins and VCP provide an extended pathway from the luminal substrate-binding lectins OS-9 and XTP3-B to cytoplasmic VCP. The third connection is a direct link between this E3 and the 26S proteasome.

The gp78 submodule seems to connect to the ERAD network through UBA1. This protein interacts with UBXD8 and Derlin-1/2, and has a functional polyubiquitin-binding domain, indicating that it may function as a membrane nexus integrating ubiquitin conjugation, dislocation and extraction. A VCP-binding site within the cytoplasmic domain of gp78 (ref. 42) means that this complex can associate with VCP in at least two ways. VCP interacts directly with multiple compo-
nents of the ERAD machinery including Derlin-1 and Derlin-2 (refs 27, 59,64), VIMP (refs 26,27), gp78 (ref. 42), UBXD2 (ref. 65) and UBXD8 (refs 66,67). With at least six different recruitment sites for VCP within the ERAD network, it is not surprising that disruption or silencing of this cytoplasmic AAA+ ATPase has a more universal effect on the degradation of diverse ERAD substrates when compared with the loss of an individual factor (Fig. 4f). Multiple recruitment avenues at the ER membrane may reflect an acquired adaptability of VCP to accommodate and engage the diverse substrates it encounters. Moreover, VCP accessory factors (for example, UBE4A, VCIP135 and SVIP), functionally essential for specific substrates (Fig. 4f), could confer an added level of specificity or may reflect a requirement for different VCP configurations at different steps of the dislocation and membrane extraction processes.

`Input’ of luminal substrates into the Hrd1 submodule occurs through the well-established interaction with SEL1L. A capacity of the Hrd1 submodule to engage substrates independently of SEL1L is also supported by several observations: SEL1L is dispensable for GluR1 degradation (Fig. 4f); Hrd1 overexpression enhanced GluR1 degradation while stabilizing TTR

We speculate that, given its close interaction with Hrd1, FAM8A1 with some ERAD substrates being partially stabilized by knockdown of this work was supported by grants from the NIH to R.R.K. and J.W.H. J.C.C. was supported by NRSA fellowships from NIH. E.J.B. was supported by a version of the paper at http://www.nature.com/naturecellbiology

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AUTHOR CONTRIBUTIONS

The manuscript was written collectively by J.C.C., J.A.O. and R.R.K. Experiments and data analysis were carried out by J.A.O. and J.C.C. with assistance from C.M.R. R.E.T. and E.J.G. LC-MS/MS analysis was carried out by T.A.S. CompPASS analysis was carried out by M.E.S. and E.J.B with support from J.W.H.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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METHODS

Plasmids and constructs. ERAD component complementary DNAs (Supplemental Table S10) were cloned into the pCDNA3.1 vector in frame with an S-tag (KETAAAKFERQHMDS) either at the N or C terminus. For some, the endogenous targeting signal was replaced with the S-tagged ERAD component was selected by G418 resistance and limiting dilution. Clonal HEK293 cell lines expressing GFP-fusion ERAD reporters are described below.

Bait selection. Primary baits were selected on the basis of previous reports or on orthology to known yeast ERAD components. Secondary baits were selected using multiple criteria, including: identification as an HCIP with several primary baits; identification as an HCIP in both digitonin and Triton X-100; presence in LC–MS/MS analyses with high total spectral counts; a predicted ER localization; and a domain structure or previous reports suggesting a potential function of relevance to ER quality control.

Mass spectrometry. Cells were collected in PBS and solubilized in lysis buffer (50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA) containing Complete protease inhibitor cocktail (Roche), and either 1% digitonin or 1% Triton X-100. Lysates were spun twice, first at 1,000g and the supernatant was resuspended at 20,000g. A quantity of 1–1.5 mg of total protein from cleared lysates was affinity purified with S-protein (Novagen). Bead-bound complexes were washed three times in lysis buffer containing 0.1% digitonin or Triton X-100 then twice in 50 mM ammonium bicarbonate, at pH 8. Bound proteins were eluted by overnight treatment with Ripagent (Waters) and subjected to trypsin digestion (Promega) before injection into the mass spectrometer. Samples were analysed in duplicate/triplicate on a system consisting of a CTC-PAL autosampler (Leap Technologies), a capillary gradient HPLC pump (Agilent Model 1100) and a linear ion-trap mass spectrometer (Model LTQ, ThermoFisher Scientific). The acquired MS/MS spectra were searched using the MASCOT protein database search program (Matrix Science) against a full database of human protein sequences to which the set of S-tagged protein sequences and S-protein sequence were added.

Data deposition. The spectral files reported in this article have been deposited in the Proteome Commons Tranche repository (http://tranche.proteomecommons.org) and can be accessed using the following hash: ZPkwz2xi + VY1M2UG0B409uKH7Mcr0XkFam9/xha4QUN/6O5 + K/1+NnuuEmibJPsWi/d/1V/FjVsYbrq08nDys7f8AAAABA4XBA +=.

CompPASS analysis. A MASCOT-generated list of all interacting proteins and their corresponding total spectral counts from duplicate MS analyses for each bait affinity complex was merged with a database containing 102 unique baits analysed previously4. CompPASS output metrics were then adjusted using both a weighting factor and normalization such that a WD5 score greater than 1 signifies a HCIP. Briefly, the CompPASS-calculated WD score assesses interacting protein abundance (peptide number), uniqueness (number of baits that interact with the protein) and replication (number of experiments the interaction is observed in) to determine HCIPs (ref. 21). To analyse data sets containing high numbers of shared interactors (for example, autophagy network and ERAD network), a normalized WD score (\( WD^N \)) was developed and has been described in extensive detail previously22. This score includes a normalization factor based on the standard deviation of the scan number for the interactors across the bait proteins, which was found to correlate with bona fide interacting proteins22. All MASCOT and CompPASS data can be accessed through INERAD, an interactive web-based portal at http://falcon.hms.harvard.edu/impsmsdbs/compass.html. The hierarchically clustered heat map representing HCIP data was generated using MultiExperimental Viewer v4.7. It should be noted that the high WD5 score for the bait protein could be due to self-identification or a self-interaction (Fig. 1 and Supplementary Tables S2 and S3) and these two possibilities could not be distinguished in the present study. HCIP clusters were selected manually by encompassing the largest number of HCIPs proximal to each bait with a minimum of two HCIPs.

ERAD shRNA library. Target gene selection was based on a reported/suspected role in ERAD or on identification as an HCIP in proteomic analyses. shRNA target sequences were selected from the literature, the RNAi codex online repository, or generated using siRNA selection programs as indicated (Supplementary Table S7). shRNAs were cloned into the pSUPERSTAR expression vector23. The library contains 309 shRNA constructs, including 3 negative control constructs, 222 constructs targeting 45 reported ERAD components, and 87 constructs targeting 14 potential ERAD components. Each gene is targeted by an average of ~5 shRNAs.

Cell culture, transfection and stable cell lines. HEK293 and HeLa cells were maintained in DMEM (Mediatech) +10% animal serum complex (Gemini Bio-Products) at 37 °C and 5% CO2. Cells were transfected by the calcium-phosphate co-precipitation technique or with FuGENE6 (Roche). Stable HEK293 clones/ pools expressing ERAD component were selected by G418 resistance and limiting dilution. Clonal HEK293 cell lines expressing GFP-fusion ERAD reporters are described below.

Functional genomic analysis of the ERAD network. Clonal HEK293 cell lines expressing GFP-tagged substrates (GFP\(^+\)), GFP–Glur1, TTR\(^{D18G}\)–GFP, A1AT\(^{NHK}\)–GFP, A1AT\(^{NHK}\)–GFP and GFP–CFTR\(^{F508}\) were obtained by G418 selection followed by limiting dilution and/or sorting by FC and expressed previously23. Cell line selection was based on multiple criteria including: expression of full-length protein; a single GFP peak as measured by flow cytometry; and accumulation with MG132 (10 μM for 12 h). For primary screening, reporter cells were seeded in 96-well plates (15,000 cells per well) and reverse-transfected with FuGENE6 with individually arrayed pSUPERSTAR-shRNA plasmids (175 ng) and pCDNA3–mCherry (CHFP) (25 ng). After 72 h, cells were analysed using high-throughput flow cytometry (LSR-II, Becton Dickinson). Mean GFP fluorescence intensity was determined for ~2,000 CHFP-positive cells and normalized to the mean GFP fluorescence intensity of pSUPERSTAR empty vector (\( n = 3 \)). Potential positive hits (Z scores > 1.5) were re-screened through all reporters, and the mean GFP fluorescence intensity for 20,000 cells was measured. shRNAs scoring positive in three independent experiments and also depleted for the target transcript were considered hits. The heat map (Fig. 4f) used mean GFP fluorescence values for each shRNA that were normalized to values resulting from a 3 h MG132 treatment, so as to facilitate comparison of each shRNA effect between reporter cell lines.

Immunopurification, affinity purification and immunoblotting. Cells were collected and affinity purified (S-protein) as described above. For immunoprecipitation (IP) and affinity purifications were performed using the Pierce direct immunoprecipitation kit (Thermo Scientific) and incubated with cell lysates. All samples were washed three times in lysis buffer, resuspended in Laemmli buffer +10 mM dithiothreitol, separated by SDS–PAGE and transferred to PVDF membrane for western blotting.

Antibodies. Experiments used the following antibodies: anti-S-tag (1:5,000), anti-myc (9E10; 1:2,000), anti-Hrd1 (1:50; gift from R. Wojcikiewicz, SUNY Upstate Medical University, USA), anti-SEL1L, anti-UBR6, anti-AUP1 (gifts from H. Ploegh, Whitehead Institute, USA), anti-VCP (1:1,000; Novus), anti-HA (12CA5; 1:1,000), anti-KDEL (1:500; Stressgen), anti-calnexin (1:500; Assay Designs), anti-GFP (1:1,000; Roche), anti-tubulin (gift from T. Stearns, Stanford University, USA) and anti-ubiquitin (FK2; 1:2,000; BioMol). The polyclonal rabbit anti-FAM8A1 (Abcam; 1:1,000) and pcDNA3.1(-) (gift from R. Malinow, University of California, San Diego, USA). GFP–CFTR\(^{F508}\) was subcloned into pcDNA3.1 vector in frame with an antibody to the N-terminus (A1AT\(^{NHK}\) (175 ng) and pcDNA3–mCherry (CHFP) (25 ng). After 72 h, cells were analysed using high-throughput flow cytometry (LSR-II, Becton Dickinson). Mean GFP fluorescence intensity was determined for ~2,000 CHFP-positive cells and normalized to the mean GFP fluorescence intensity of pSUPERSTAR empty vector (\( n = 3 \)). Potential positive hits (Z scores > 1.5) were re-screened through all reporters, and the mean GFP fluorescence intensity for 20,000 cells was measured. shRNAs scoring positive in three independent experiments and also depleted for the target transcript were considered hits. The heat map (Fig. 4f) used mean GFP fluorescence values for each shRNA that were normalized to values resulting from a 3 h MG132 treatment, so as to facilitate comparison of each shRNA effect between reporter cell lines.

Measurement of UPR induction of the ERAD network. HEK293 cells were transfected with the expression plasmid encoding S-tagged target protein along with the corresponding control or shRNA-targeting plasmid at a ratio of 1:3. Levels of the S-tagged target protein were subsequently analysed by immunoblotting of either SDS–solubilized cell lysates or of S-protein agarose affinity-purified proteins.

Measurement of UPR induction of the ERAD network. HEK293 cells were transfected with the expression plasmid encoding S-tagged target protein along with the corresponding control or shRNA-targeting plasmid at a ratio of 1:3. Levels of the S-tagged target protein were subsequently analysed by immunoblotting of either SDS–solubilized cell lysates or of S-protein agarose affinity-purified proteins.

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using the Pfaffl method with β-actin as the reference gene. Primer sequences are listed in Supplementary Table S9.

**Immunofluorescence** microscopic analysis of protein localization and topology. HeLa cells grown on poly-L-lysine-coated glass coverslips were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 and blocked by 1% bovine serum albumin. Primary antibody incubation (2 h at room temperature) was followed by incubation with AlexaFluor secondary antibodies (Invitrogen, 1 h at room temperature). Nuclei were stained with 10 μg ml⁻¹ bisbenzamide for 10 min before mounting. For analysis of protein topology, HeLa cells grown and fixed as above were permeabilized with either 20 μM digitonin for 1.5 min or 0.1% Triton X-100 for 3 min at room temperature to allow permeabilization of the plasma membrane or permeabilization of both the plasma and ER membranes, respectively. Cells were washed three times with PBS and immunostained as described above. Stained cells were visualized on a Zeiss Axiovert 200M (×40 air objective) and the resulting images were acquired digitally (Roper Scientific).

**Sucrose gradient fractionation.** HEK293 cells were lysed as described above. A quantity of 1.5 mg of total protein from cleared lysates was adjusted to 5% sucrose and loaded onto 10–40% continuous sucrose gradients (containing 0.1% digitonin) prepared using a Gradient Master (BioComp). Samples were centrifuged in an SW41 rotor at 39,000 r.p.m. for 14.25 h at 4°C. Fractions of 1 ml in volume were collected, sucrose concentrations were adjusted to approximately 20% with lysis buffer, and S-tagged proteins were affinity purified with S-protein agarose.

**Metabolic labelling and pulse-chase assay.** Pulse-chase assays of GFP–GluR1 reporter cells were carried out as described previously.

**Proteomic interaction data set analysis.** LC–MS/MS analysis of replicate affinity-purified protein complexes from 25 bait proteins identified a total of 10,481 proteins in digitonin and 8,262 in Triton X-100. Approximately two-thirds were redundant entries, with the number of proteins having unique GenInfo Identifier numbers reduced to 3,325 (digitonin, 31.7%) and 2,917 (Triton X-100, 35.9%) for the entire analysis. A total of 320 (digitonin) and 202 (Triton X-100) entries were designated as HCIPs by CompPASS (WD-score > 1) and included redundant entries representing shared interactors (Supplementary Tables S2 and S3). A breakdown of digitonin and Triton X-100 HCIPs is presented in Supplementary Table S4, including specific analyses for primary and secondary baits. The total number of CompPASS-identified HCIPs represents all bait-interacting proteins. Bait proteins often had WD-score > 10, but as we could not distinguish bait from an endogenous, homo-oligomerized counterpart, HCIPs representing their identical bait proteins were not considered (bait self-identification), neither were trypsin (introduced during the experimental method (trypsin)) and proteins pulled down non-specifically by S-protein agarose from untransfected HEK293 cells (bait control). The result yielded 267 (digitonin) and 153 (Triton X-100) interactions (cellular HCIPs/ERAD interactions) with 143 (digitonin) and 97 (Triton X-100) corresponding to unique HCIPs. Proteins whose gene ontology assignments were cytoskeletal, mitochondrial, peroxisomal or nuclear (for example, MYH9, KRT8, PEX19, TOMM20 and MCM2) were deemed unlikely to be ERAD-related and removed from subsequent analysis (likely false positives) and indicated as such in individual interactomes (Supplementary Fig. S4). To determine the number of new HCIPs identified, all primary and secondary baits were subtracted (other primary/secondary baits as prey), as well as proteins implicated in ERAD (for example, VCP, GRP94 and proteasome subunits) but not used as bait (reported ERAD factors). Ultimately, we identified 59 (digitonin) and 30 (Triton X-100) uncharacterized proteins of interest with a wide range of topologies and functional domains, and a potential role within the ERAD network (Supplementary Fig. S3d, not including the 10 secondary baits).

**Comparison and integration of proteomic data set with reported interactions.** A list of reported pairwise interactions for ERAD components from published data was manually curated and used to generate a more comprehensive picture of the mammalian ERAD interaction network (Supplementary Table S5). This table includes: an assigned ‘interaction number’; bait and prey defining the interaction and whether our study used the bait (red); the method(s) of identification (for example, affinity capture-MS, two-hybrid, and so on) and the relevant reference(s) for the reported interaction; whether the reciprocal interaction was shown and its corresponding ‘interaction number’; detection of the reported interaction in our digitonin or Triton X-100 interaction networks; and the presence of this interaction in the STRING online protein interaction database. STRING lists only 33/131 reported interactions (~25%), supporting the use of a manually curated list rather than an online resource. Of the 81 reported interactions using the 25 primary/secondary baits, 31 were confirmed by our proteomic analysis with an additional 3 being identified in a reciprocal interaction not previously reported. Seven more reported interactions were observed but were subthreshold (WD-score < 1). We identified 21 reciprocal and 36 unidirectional interactions between baits and ERAD components, and interactions with 71 uncharacterized proteins of interest that scored above the threshold for HCIP classification (Supplementary Table S5 and Fig. S3d). Combining reported pairwise interactions (Supplementary Table S5) with INiERAD (Fig. 2) and the RNAi-mediated ‘epistasis-like’ experiments (Fig. 3) yielded an up-to-date, comprehensive view of the ERAD interaction network (Supplementary Fig. S5).

71. Ward, C. L., Omura, S. & Kopito, R. R. Degradation of CFTR by the ubiquitin-proteasome pathway. Cell 83, 121–127 (1995).
**SUPPLEMENTARY INFORMATION**

**Figure S1** Strategy to elucidate functional complexes of the mammalian ERAD pathway. Flow chart illustrating the strategy used to identify functional ERAD complexes by integrating high-content proteomics, functional genomics and gene expression data sets (see Methods).

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Figure S2 Subcellular localisation of S-tagged ERAD components. HeLa cells, transiently expressing individual S-tagged ERAD components, were immunostained simultaneously with anti-S-tag (green) and anti-KDEL (red) antibodies, with nuclei identified by bisbenzamide (blue). (a) Primary baits employed for initial proteomic analyses. (b) Secondary baits identified as HCIPs from initial proteomic screening (MMGT1-S not shown).
Figure S3: Analysis of HCIP abundance and gene ontology (GO). (a) The total number of interactors (red) and identified HCIPs (blue) are plotted for individual baits solubilised in DIG (top) or TX-100 (bottom). (b) Venn diagram showing the uncharacterised HCIPs obtained in DIG (red), TX-100 (blue), or in both (purple). 28 HCIPs were identified in both detergents, with the 10 HCIPs boxed in grey selected and designated as secondary baits. (c) Individual pie graphs reflecting the relative percentage of HCIPs belonging to each designated GO annotation in DIG (top) or TX-100 (bottom). (d) Enrichment of HCIPs in GO defined categories. Determined as the ratio of the representative percentage of HCIPs vs. entire proteome.
**SUPPLEMENTARY INFORMATION**

Figure S4 Interaction maps for individual ERAD baits. Interactomes for each individual S-tagged ERAD component are shown, representing HCIPs detected in DIG only (dashed-green line), TX-100 only (dashed-purple line), and both DIG and TX-100 (solid-black line). ER-localised/ERAD-related HCIPs are indicated with red text and where appropriate, by their assigned colour from DIG and TX-100 (solid-black line). ER-localised/ERAD-related HCIPs are shown as: cytoplasmic/nuclear (grey circles, grey text), peroxisomal (beige circles, light blue text). 

HCIPs of interest are depicted as dark brown circles with either red or black text, depending on their predicted localisation. HCIPs with alternative Qo localisation (i.e. non-ER) and suspected non-specific contaminants are shown as: cytoplasmic/nuclear (grey circles, grey text), peroxisomal (beige circles, green text) and mitochondrial (light brown circles, light blue text).
**Figure S5** Integration of previously reported interactions with the determined ERAD network. The previously reported interactions (Table S5) for ERAD baits and HCIPs were mapped onto the ERAD interactome from Fig. 1b with the appropriate refinements from Fig. 2. Shown are reported interactions that were identified in our study (green), interactions only identified in our study (red), and additional reported interactions (blue).
**Figure S6** Analysis of gp78 and Hrd1-associated proteasome compositions. (a, c, e, g) The calculated spectral abundance factor (Supplementary Table S6) for proteasome subunits co-precipitated with gp78 or Hrd1 in the indicated detergent is plotted. (b, d, f, h) The average spectral abundance factor for 20S core subunits (PSMA and PSMB subunits) and 19S core subunits (PSMC and PSMD subunits) co-precipitated with gp78 or Hrd1 in the indicated detergent is plotted with the standard error.
Figure S7 Unassembled GluR1 is a bona fide ERAD substrate. (a) Illustration of GFP-GluR1 fusion protein topology. (b) Colocalisation of GFP-GluR1 (green) expressed in HEK293 cells with the ER marker calnexin (red). Nuclei are stained with DAPI (blue). (c) HA-GluR1 migration in the absence and presence of Endo H and MG132 (10 μM). EndoH sensitivity indicates that GluR1 is retained in the ER in its core glycosylated form and proteasome inhibition does not promote its maturation. (d) GFP-GluR1 pulse-chase assay. A t1/2 for GluR1 was calculated to be ~2.5 hrs. and its degradation is mitigated by MG132. (e) GluR1 is ubiquitinated upon addition of MG132. GFP-GluR1 was immunoprecipitated with anti-GFP antibody, and Western blots were probed with anti-Ub (top) and anti-GFP antibodies (bottom). (f) Fluorescence histograms of the GFP-GluR1 stable cell line untreated (CTRL) and treated with MG132 (6 hrs, 10 μM) as measured by flow cytometry (left). Time course of GFP-GluR1 degradation after treatment with MG132 (10 μM) and/or emetine (10 μM) over 12 hrs (right).
**Supplementary Information**

**Validation of shRNA knockdown by Western blotting and qRT-PCR.** To determine the efficacy of shRNA constructs targeting the each gene, plasmids encoding the corresponding S-tagged or myc-tagged target protein were transiently co-expressed in HEK293 cells with a control shRNA plasmid targeting firefly luciferase or shRNA plasmids targeting potential ERAD components (indicated by the library reference number; Table S7a). SDS-solubilised cellular lysates (a-c) or S-protein agarose affinity-purified proteins from TX-100 lysates (d-v) were separated by SDS-PAGE and analysed by Western blotting with anti-S-peptide or anti-myc antibodies and anti-tubulin antibodies as a loading control. (w) To determine the efficacy of shRNA constructs at depleting the target transcript, a control shRNA plasmid targeting firefly luciferase or shRNA plasmids targeting potential ERAD components (indicated by the library reference number; Table S7a) were transiently expressed in HEK293 cells. Transcript levels for the indicated components were determined by qRT-PCR, normalised to cells transfected with the control plasmid targeting firefly luciferase and to the percentage of transfection, and presented as the normalised expression level.
**Figure S9** Integrated substrate-specific degradation snapshots. The normalised fold change in mean GFP fluorescence of ERAD reporter lines in response to shRNA-mediated knockdown of ERAD components represented as a heat map (Fig. 3f) was directly mapped onto the comprehensive ERAD interactome (Fig. S5) to construct an integrated snapshot of substrate-specific functional ERAD networks for two example substrates, TTR(D18G)-GFP (panel (a)) and GFP-GluR1 (panel (b)).
**A**

GFPu Integrated degradation map

**B**

CFTR(ΔF508) Integrated degradation map

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**Figure S10** Integrated substrate-specific degradation snapshots for GFPu and CFTR(ΔF508). Integrated snapshots of substrate-specific functional ERAD networks as described in Fig. S9 are shown for GFPu (a) and CFTR(ΔF508) (b).
Figure S11 Integrated substrate-specific degradation snapshots for A1AT(NHK) and A1AT(NHK-QQQ). Integrated snapshots of substrate-specific functional ERAD networks as described in Fig. S9 are shown for A1AT(NHK) (a) and A1AT(NHK-QQQ) (b).
Figure S12 Full scans of immunoblots.
Figure S12  Full scans of immunoblots continued
Figure S12  Full scans of immunoblots continued
Figure S12 Full scans of immunoblots continued
Figure S12 Full scans of immunoblots continued
Supplementary Tables

**Table S1** Listing of primary and secondary bait proteins. Primary (top) and secondary (bottom) S-tagged proteins employed as baits in the proteomic analyses are shown with relevant information including alternative names, known functions, protein domains/repeats, cellular localisation (ER-L: ER luminal, ER-M: ER integral membrane, C: cytoplasmic), and identified yeast orthologs (Standard and Systematic name) are also shown.

**Table S2** CompPASS-identified HCIPs and complete MASCOT list of interactors in DIG. (a) Listing of proteins interacting in DIG that displayed a WDN-score above the threshold score of 1 and were therefore designated as HCIPs. Also shown are additional statistical measures of interactor confidence, including Z-score, WS-score, and ZD-score. Self-identification of the bait protein identified is shown in red. (b) Listing of all MASCOT-identified protein interactors and their respective number of peptide scans found in affinity purifications of bait proteins from DIG-solubilised cell lysates.

**Table S3** CompPASS-identified HCIPs and complete MASCOT list of interactors in TX-100. (a) Listing of proteins interacting in TX-100 that displayed a WDN-score above the threshold score of 1 and were therefore designated as HCIPs. Also shown are additional statistical measures of interactor confidence, including Z-score, WS-score, and ZD-score. The bait protein identified in each purification is shown in red. (b) Listing of all MASCOT-identified protein interactors and their respective number of peptide scans found in affinity purifications of bait proteins from TX-100-solubilised cell lysates.

**Table S4** Analysis of proteomic interaction data. (a) Analysis and breakdown of proteomic data obtained in DIG. The total number of HCIPs identified by CompPASS is shown as well as the number of scans contributed by Bait self-identification, Trypsin, and Bead controls that were removed to yield the total number of HCIPs/ERAD interactions. The total number HCIPs is further broken down into likely false positives, primary / secondary baits obtained as prey, reported ERAD factors, and uncharacterised proteins of interest. The number of HCIPs is also shown separately for primary and secondary baits, as well as the overlap between these two data sets. (b) Same as in panel (a), but for data obtained in TX-100.

**Table S5** Previously reported ERAD component interactions. Listing of reported pairwise interactions. Each interaction between a bait and prey was assigned an ‘interaction number’. The method by which the interaction was identified and the relevant reference (blue text) are indicated. Proteins used as baits in this study are shown (red text), and gray shading indicates proteins that were not employed as baits or identified as HCIPs in this study. The identification of the reported interaction in our DIG or TX-100 analyses or in the STRING database is indicated as an ‘O’ (identified) or as an ‘X’ (not identified). A comparative analysis of the reported interactions literature and those identified in our study is shown in the inset table.

**Table S6** E3-proteasome subunit interactions. Listing of all MASCOT-identified proteasome subunits, their number of peptide scans, and their spectral abundance factor (SAF) from affinity purifications of the E3s Hrd1 and gp78 in DIG or TX-100 as indicated. Proteasome subunits determined by CompPASS to be HCIPs (WDN-score > 1) are highlighted in blue.

**Table S7** Raw substrate fluorescence data from the primary ERAD reporter screen. (a) Complete listing of all shRNA target sequences, library reference numbers, and their effects on the fluorescence levels of the ERAD reporter cell lines tested in the initial round of screening, including GFPp, GFP-GluR1, TTR(D18G)-GFP, A1AT(NHK)-GFP, and GFP-CFTR(ΔF508). (b) shRNA constructs employed in the second round of screening in the following cell lines: GFPp, GFP-GluR1, TTR(D18G)-GFP, A1AT(NHK)-GFP, A1AT(NHK-QQQ)-GFP, GFP-CFTR(ΔF508), and GFP cell lines. These data are represented as a heat map in Fig. 4f.

**Table S8** ERAD component data and links. Listing of ERAD components and identified HCIPs of interest along with relevant information, including gene and protein names, known function, localisation, UNIPROT ID, and known yeast orthologs.

**Table S9** qRT-PCR primers. Listing of qRT-PCR primer sequences used to assess the affect of UPR induction on transcript levels in Fig. 5 and to assess shRNA knockdown of target transcript in Supplementary Fig. S8.

**Table S10** ERAD cDNA sources. Listing of cDNA sources for expression of the bait proteins employed in this study.