Conserved degronome features governing quality control-associated proteolysis

Bayan Mashahreh[1], Shir Armony[1], Kristoffer Enøe Johansson[3], Alon Chappleboim[1], Nir Friedman[1], Richard G. Gardner[2], Rasmus Hartmann-Petersen[3], Kresten Lindorff-Larsen[3], Tommer Ravid[1*]

[1]Department of Biological Chemistry, The Alexander Silberman Institute of Life Sciences, The Hebrew University of Jerusalem, Jerusalem, Israel

[2]Department of Pharmacology, University of Washington, Seattle, WA 98195, USA

[3]The Linderstrøm-Lang Centre for Protein Science, Department of Biology, University of Copenhagen, Copenhagen, Denmark

*Correspondence: tommer.ravid@mail.huji.ac.il

Summary

The eukaryotic proteome undergoes constant surveillance by quality control systems that either sequester, refold, or eliminate aberrant proteins by ubiquitin-dependent mechanisms. Ubiquitin-conjugation necessitates the recognition of degradation determinants, termed degrons, by their cognate E3 ubiquitin-protein ligases. To learn about the distinctive properties of quality control degrons, we performed an unbiased peptidome stability screen in yeast. The search identified a large cohort of proteome-derived degrons, some of which exhibited broad E3 ligase specificity. Consequent application of a machine-learning algorithm established constraints governing degron potency, including the amino acid composition and secondary structure propensities. According to the set criteria, degrons with transmembrane domain-like characteristics are the most probable sequences to act as degrons. Similar quality control degrons were identified in viral and human proteins, suggesting conserved degradation mechanisms. Altogether, the emerging data indicate that transmembrane domain-like degron features have been preserved in evolution as key quality control determinants of proteins' half-life.

Keywords: Protein Quality Control, Quality Control Associated Proteolysis, Ubiquitin-Proteasome System, degrons, Global Protein Stability, Protein Stability Index, peptidome, degronome.

Introduction

Intracellular protein quality control (PQC) is a principal regulatory mechanism for the maintenance of protein homeostasis (Balchin et al., 2016). PQC systems continuously survey the proteome and execute a triage of unfolded protein states, the result of which is either refolding or, if beyond repair, sequestration or degradation of aberrant proteins (Kästle and Grune, 2012; Shiber and Ravid, 2014). Protein refolding and sequestration are primarily mediated by molecular chaperones (Kim et al., 2013; Sontag et al., 2017; Tyedmers et al., 2010), while the Ubiquitin-Proteasome System (UPS) executes quality control-associated proteolysis (QCAP) (Amm et al., 2014).

A key to understanding protein homeostasis is deciphering the mode by which QCAP pathways discern the folding state of proteins. It has been established that the ubiquitin conjugation system, via E3 ubiquitin-protein ligases and auxiliary chaperones, recognize degradation determinants termed degrons (Varshavsky, 1991) that constitute inherent sequences and structural features, as well as acquired post-translational modifications (Guharoy et al., 2016). To date, degrons have been mostly identified through studies of regulated protein degradation mechanisms, such as those involved in cell division and differentiation, as well as in many cancer-related diseases (Koliopoulos and Alfieri, 2021; Mészáros et al., 2017; Rape, 2018). These studies identified inherent degrons as short motifs, such as the destruction box of cyclins, as well as acquired degrons that are activated by phosphorylation or other post-translational modifications (Ang and Wade Harper, 2005; Davey and Morgan, 2016; Filipčík et al., 2017; Lucas and Ciulli, 2017). However,

the repertoire of known degrons cannot explain the large diversity in half-lives exhibited by the proteome (Christiano et al., 2014; Eden et al., 2011).

Our earlier work exposed the large sequence heterogenicity of the cellular degron landscape (degronome) in yeast, which led to the proposition that the majority of the eukaryotic proteome contains cryptic QCAP degrons that may become exposed naturally or under misfolding conditions, such as cellular and environmental stresses (Ella et al., 2019; Geffen et al., 2016). These degrons target protein ubiquitination via the activity of a relatively small number of designated QCAP E3 ligases, suggesting that each recognizes a large set of substrates (Breckel and Hochstrasser, 2021). Furthermore, QCAP E3 enzymes can act redundantly in the ubiquitination of their substrates, seemingly exhibiting overlapping recognition mechanisms (Hickey et al., 2021; Maurer et al., 2016; Samant et al., 2018). However, the significance of this functional redundancy is not yet fully understood.

Here we describe a yeast-adapted Global Protein Stability (GPS)-peptidome technology (yGPS-P) originally established for the discovery of degrons in mammalian cell lines (Koren et al., 2018; Lin et al., 2018; Yen et al., 2008). By employing a peptide library fused to yGPS-P, we have identified multiple degron sequences that were subsequently analyzed using a machine learning algorithm. The resulting computer program, termed Quality Control Degron Prediction (QCDPred)(Johansson et al., 2022) revealed amino acid and secondary structure preferences in QCAP degrons. The determined degron features were highly dependent on the overall hydrophobicity, and consistently transmembrane domains (TMDs) exhibit extreme degron potency, signifying their critical role in the degradation of integral membrane proteins prevented from entering the secretory pathway.

Results

Yeast-based GPS-peptidome technology

To set up a comprehensive degron discovery platform in the yeast *Saccharomyces cerevisiae* proteome, we applied a fluorescence-based GPS technology (Yen et al., 2008), previously developed in cultured human cells (Koren et al., 2018; Lin et al., 2018). yGPS-P utilizes a bicistronic gene expression system in which codon-optimized versions of yeast enhanced Cherry and yeast enhanced GFP are expressed from a single transcript. Yet, the two proteins are translated separately due to the presence of an Internal Ribosome Entry Site (IRES) upstream to GFP that allows translation initiation in a cap-independent manner (Edwards and Wandless, 2010) (**Figure 1A**). A yeast GPS peptidome library (yGPS-P_{lib}) is generated by subsequent in-frame insertion of proteome-derived DNA fragments downstream to GFP in a yGPS-P vector (**Figure 1A**). The plasmid library is transformed into yeast, followed by quantitative flow cytometry or Fluorescence-Activated Cell Sorting (FACS) (**Figure 1B**). As both Cherry and GFP are expressed from a single transcript yet translated independently, Cherry levels reflect the basal expression of the reporters while the ratio between GFP to Cherry (shown henceforth as yeG/yeC) determines the relative GFP protein level that is governed by the fused peptide.

To validate the use of yGPS-P as a degron discovery platform, we examined the competence of two well-characterized QCAP degrons of the Doa10 E3 ligase, *CL1* and *DegAB* (Alfassy et al., 2013; Furth et al., 2011; Gilon et al., 1998, 2000) to trigger degradation of the otherwise stable GFP. Doa10 is an endoplasmic reticulum (ER)-embedded enzyme that operates in ER-associated degradation (ERAD) (McCracken and Brodsky, 1996; Swanson et al., 2001). When the yeG/yeC ratio in cells expressing the fused degrons was compared to empty vector control, a more than 5-fold decrease was observed, presumably due to UPS-mediated proteolysis (**Figure 1C**, *upper panel*). An increase in yeG/yeC in *doa10*Δ cells confirmed this assertion. That the increase in

yeG/yeC is indeed a result of elevated GFP protein levels was demonstrated by immunoblot analysis of the corresponding fusion proteins (**Figure 1C**, *lower panel*).

As a source for a peptide library, we chose a subset of proteins, all components of multimeric protein complexes that potentially undergo QCAP triage (Padovani et al., 2022; Pla-Prats and Thomä, 2022). In total, 335 yeast proteins that are part of 23 different complexes were selected (**Supplemental Table 1**). Consequently, 51-mer DNA fragments with 36-mer DNA overlaps (corresponding to 17 amino acid-length tiled peptides with 12 amino acid overlaps) were synthesized to give rise to yGPS-P_{lib} (library) containing approximately 29,500 DNA fragments. yGPS-P_{lib} was transformed into yeast, followed by flow cytometry determination of the yeG/yeC ratio. The observed decrease in yeG/yeC in yGPS-P_{lib} (**Figure 1D**) indicates the presence of a destabilizing peptide population within the tested peptidome. To assess the contribution of degrons of the UPS to GFP destabilization, the effect of the irreversible proteasome inhibitor Bortezomib (BZ) on yeG/yeC was determined. To this end, yGPS-P_{lib} was transformed into cells lacking the multidrug transporter *PDR5 (pdr5*Δ) to increase drug sensitivity (Leppert et al., 1990). Comparing mock- and drug-treated cells, we observed an increase in the overall cellular levels of ubiquitin conjugates (confirming proteasome inhibition), as well as a mild increase in yeG/yeC (**Figure 1E**, *left & middle panels*). A larger increase of yeG/yeC was observed when a pre-sorted top 10% degron-enriched population was tested (**Fig. 1E**, *right panel*, and **Supplemental Figure 1**), confirming that changes in yeG/yeC accurately reflect susceptibility to UPS-mediated degradation.

<u>Mapping QCAP degrons using a machine-learning-based approach</u>

To classify degron sequences within yGPS-P_{lib}, mid-log-phase cells expressing the appended peptidome were separated by FACS into four gates according to yeG/yeC, each containing an equal cell number, and the identity and amounts of the peptides DNA in the different gates were

determined by Next-Generation Sequencing (NGS) (**Figure 2A**). After filtering ambiguous peptides DNA from the NGS data, the contribution of 23,600 peptides to GFP stability was calculated based on their abundance in the different gates and each was assigned a Protein Stability Index (PSI) score (Yen et al., 2008)(**Figure 2A and Supplemental Table 2**). Overall, 9.5% of the analyzed peptidome had PSI values < 1.7 (on a scale of 1 – 4) (**Figure 2A)**.

Employing various search tools, we could not observe linear amino acid sequence motifs or C-terminal amino acid preferences within the examined degrons, (see also (Geffen et al., 2016). We, therefore, opted to employ a machine-learning algorithm that we have established by using the PSI data to train a logistic regression model (Johansson et al., 2022). QCDPred provided each amino acid with a unique value, based on its predicted contribution to degron potency (Johansson et al., 2022). Consequently, protein stability maps were formed for all proteins comprising the tested library that attained 100% coverage (N = 306), each includes experimental PSI values for each peptide, average PSI values for each amino acid, and QCDPred probability scores (**Supplemental Figure 2**).

To experimentally validate the high-throughput procedure and test the QCDPred model, we selected ten peptide sequences that we studied- one at a time -using the same reporter system and flow cytometry readout (**Figure 2B, Supplemental Table 3**). Five peptides (P1–P5), which we predicted by QCDPred to have degron activity (group I), and three peptides that we predicted not to have degron activity (P6–P8; group II) were confirmed in this experiment. In contrast, two peptides (P9 and P10) were found to have some degron activity even though they were not predicted as degrons by QCDPred (group III). We also found that treating the cells with a proteasome inhibitor (Bortezomib) restored GFP fluorescence for the degron sequences,

demonstrating that the lowered fluorescence is due to proteasomal degradation, lending further support that QCDPred predicts proteasomal QCAP substrates (**Supplemental Figure 3**).

As projected from previous studies of QCAP degrons, the QCDPred scores of most amino acid types are correlated with their Kyte-Doolittle hydrophobicity scores, and indeed, all hydrophobic amino acids contributed positively to QCAP degrons' probability (**Figure 2C**). Conversely, QCDPred scores of the two negatively charged amino acids, glutamate and aspartate, were significantly lower than others, suggesting that the presence of negatively charged amino acids specifically interferes with a QCAP degron's function. This was confirmed by replacing three amino acids with either glutamate or arginine in a re-evaluated proteasome-dependent degron peptide P3 from the yeast protein Qcr9 (**Figure 2D**). We concluded that the classified QCAP degronome prefers hydrophobic residues while negatively charged amino acids are disfavored.

We next examined these assertions on a native QCAP degron from the P-type Cation-transporting ATPase Pca1, that was not in our tested degron cohort. Under standard growth conditions, Pca1 is constitutively degraded via Doa10, which recognizes a cysteine-enriched degron localized within amino acids 271-320 of the cytosolic and solvent-exposed N-terminal region of Pca1 (Adle and Lee, 2008; Smith et al., 2016). However, cadmium sensing by the degron enables Pca1 to circumvent ERAD (Adle and Lee, 2008). As this region is too long for precise analysis, we located a shorter sequence that defines the operational degron, using QCDPred, between amino acids 289-305, which was predicted by AlphaFold (Jumper et al., 2021; Varadi et al., 2022) to form an exposed helix-turn-helix structure (**Figure 2E**). Examining Pca1 steady-state levels, we observed that replacing three amino acids in the degron core (**Figure 2E**) with aspartate residues greatly stabilized the protein while replacing the same amino acids with arginine residues only showed moderate stabilization (**Figure 2F**). Importantly, mutant Pca1 still retained cadmium

sensitivity (**Figure 2F**), indicating overall structural preservation. These data confirmed our assertion that negatively charged amino acids greatly interfere with native QCAP degron's function. The data also demonstrated the capability of QCDPred to identify functional degrons within the proteome while in their physiological context.

Transmembrane domains function as QCAP degrons

Intriguingly, besides the aforementioned cytosolic degron, QCDPred also assigned remarkably high degron probabilities ($P \geq 0.93$) to Pca1 TMDs (**Figure 3A**). This was unexpected, not only because QCDPred was not programmed to consider protein topology, but also because TM proteins comprised only a minute proportion of the proteins included in the screen (1.79%) and hence their small contribution to the algorithm. The observation is perfectly in line with PQC degrons being hydrophobic (**Figure 2C**). Thus, when applying QCDPred to the entire yeast proteome, the vast majority of TMDs were assigned as degrons (**Figure 3B**). Our experimental peptidome data agree with this prediction, demonstrating that 12 out of 13 peptides localized to TMDs of the inner mitochondrial cytochrome b-c1 respiratory complex (Trumpower, 1990), the only TM-embedded complex in our peptidome cohort (**Supplemental Table 1**), function as degrons (**Figure 3C and Supplemental Figure 4**). In line with these findings, most predicted TMD degrons in the yeast proteome were enriched in the highest QCDPred score range of 0.95-1.0 while the rest of the degrons were underrepresented in this range (**Figure 3D**). Thus, according to QCDPred, TMDs comprise the most potent QCAP degron sequences. Hence, the hydrophobic sequence and possibly structural resemblance to TMDs is likely a significant feature of QCAP degrons. TMDs themselves could be relevant as PQC degrons in cases when TM proteins fail to insert correctly in membranes (see next).

Since the QCDPred algorithm is based on data from short peptides and thereby devoid of the cellular context, we wished to empirically assess the putative function of TMDs as potential degrons in a more physiological setting. To this end, three single-pass type-I proteins were selected to examine our hypothesis. These include the COPII-coated vesicles protein Erp2 (Marzioch et al., 1999), Atg27, which is involved in autophagy, and coated-vesicle transport (Yen et al., 2007), and Ksh1 that functions in the early steps of the secretory pathway (Wendler et al., 2010). When expressed as GFP fusions, all three proteins exhibited membrane localization (**Figure 4A**). QCDPred assigned degron function to both the N-terminal signal peptides (SPs) that mediate ER insertion (Hegde and Keenan, 2022) and the TMDs of the three proteins (**Figures 4B**). This was confirmed experimentally by fusing either the TM or SP regions of the three proteins C-terminally to GFP (a position that is unlikely to support ER translocation), followed by flow cytometry analysis (**Figures 4C, 4D**). Thus, besides ER targeting, SPs may additionally function as QCAP degrons if translocation fails.

As the removal of the SP would likely disrupt ER targeting, we hypothesized that this would in turn lead to a rapid turnover of TMD-containing proteins via recognition of TMDs as degrons. This was tested by determining the steady-state levels, with or without the SP, of Erp2, Atg27, and Ksh1 (**Figure 4E, Supplemental Figure 5**). Appending the full-length proteins N-terminally to GFP resulted in partial destabilization, assumingly by QCAP, that indeed, was greatly enhanced by the removal of the SP. Curiously, $_{\Delta SP}$Atg27 did not respond to proteasome inhibition while levels of $_{\Delta SP}$Erp2 and $_{\Delta SP}$Ksh1 were significantly increased (**Supplemental Figure 5**), suggesting a proteasome-independent degradation mechanism for the mislocalized autophagy-associated protein. Notably, positioning of the cytosolic exposed TMD degrons is immaterial to their function

as both N-terminally- and C-terminally-appended SP-excluded proteins were substantially unstable (**Figures 4E, 4F**).

QCAP degron characteristics

The data so far indicate that in the yeast proteome QCAP degrons are widespread and that these regions are prevalent in hydrophobic residues while negatively charged residues are scarce. Nevertheless, we found that within hydrophobic degrons there is a bias toward specific residues (**Figure 5A**): Comparing the distribution of amino acids in hydrophobic TMDs with high degron probability ($P \geq 0.85$) to a small set of TMDs with low degron probability ($P < 0.85$), we found that the former is enriched in bulky and branched hydrophobic amino acids, while the latter express small, non-polar, amino acids instead. In addition, we identified a prevalence of alpha-helical configurations in QCAP degrons in their native protein context (**Figure 5B**). This not only agrees with the established helical structure of TMDs but also correlates strongly with an increase in the probability of non-TMD degrons (**Figure 5C**). Interestingly, PQC degrons were hardly found in N/A (not assigned) regions that are likely intrinsically disordered (**Figure 5B**). This implies that for most QCAP degrons to become active, the protein must be structurally perturbed so that the degron is exposed. Indeed, we have found that for disordered proteins and regions there is a correlation between the presence of predicted degrons and the abundance and half-lives of the proteins (Johansson et al., 2022). Altogether, our data indicate that QCAP degrons are enriched in bulky hydrophobic TMD-like entities.

We next tested whether the linear order of amino acids within a degron determines its function. To this end, the amino acid sequence of a peptide P1 from the yeast protein COB of the b-c1 complex was scrambled to form two random peptides having identical amino acid content, hence also identical degron probability (**Supplemental Table 3**). When fused to the C-terminus of GFP,

all three peptides were predicted to form helices, however to various degrees (**Supplemental Figure 6**). Both scrambled peptides not only conferred GFP degradation but were even more potent than the original P1 peptide, indicating that the general chemical properties of the degron, rather than its exact linear sequence is a principal QCAP degron determinant (**Figure 5D**).

Degron presence correlates with proteins function

We next searched for roles governing the distribution of degrons in the proteome. Specifically, whether it is simply dependent on the probability of finding a defined sequence within the entire proteome or associated with specific protein property or function. As shown in **Figure 5E**, a weak monotonic relation was observed between protein length and degron presence (Spearman rank correlation coefficient (ρ) of 0.46), thus excluding random distribution of the QCAP degronome. We then analyzed degrons through Gene Ontology (GO) annotation (**Figure 5F**). To this end, the yeast proteome was classified, based on QCDPred score into two groups- one with and one without degrons at high significance ($P \geq$ or < 0.85). Each group was then used as input for determining GO processes using the Saccharomyces Genome Database (SGD) Gene Ontology Slim Term Mapper (https://www.yeastgenome.org/goSlimMapper). Data in **Figure 5F and Supplemental Figure 7** show that proteins engaged in transport and lipid metabolism are enriched in QCAP degrons. Many of these protein classes are integrated into membranes, an observation that agrees with our finding that TMDs can also act as QCAP degrons when exposed to the PQC system. Conversely, proteins, where QCAP degrons are underrepresented, are mostly involved in transcription, translation, ribosome assembly, as well as protein folding. The latter group is of particular interest as it implies that the exclusion of QCAP degrons in chaperones involved in proteolysis renders them resistant to degradation themselves. Indeed, QCDPred analysis of a collection of cytosolic/nuclear yeast chaperones predicted one or more degrons in Hsp90, Hsp104,

and Hsp110 family members, while Hsp40 and Hsp70 family members, that are directly involved in proteolysis (Shiber and Ravid, 2014), were mostly devoid of QCAP degrons (**Supplemental Figure 8**).

Partial selectivity of QCAP E3 ligases

To learn about UPS-dependent QCAP functionality, we next examined degron specificity of Ubiquitin-protein E3 ligases. To this end, the aforementioned peptide library was inserted into yeast strains lacking one of three well-defined QCAP E3 ligases – Doa10, which is a multi-TMDs E3 ligase that localizes to the outer leaflet of the ER membrane and the nuclear envelope (Deng and Hochstrasser, 2006), Ubr1, which is a soluble protein residing in the cytoplasm and the nucleoplasm (Prasad et al., 2018), and San1, which is exclusively nuclear E3 (Gardner et al., 2005). yGPS-P_{lib} transformation into E3 deleted (*E3*Δ) strains was followed by FACS and NGS, and PSI scores of degrons having $P < 1.7$ were determined and compared to that of *pdr5*Δ cells that served as a control strain (**Supplemental Table 2**). All knockout groups displayed a significant increase in degrons PSI scores (Kruskal-Wallis test $P < 0.001$). A Venn diagram determining E3 functional overlaps indicates that approximately one-third of degrons were recognized by two or more E3 enzymes (**Figure 5G**). This finding is consistent with that of Hickey and colleagues who showed distinct yet overlapping QCAP E3 ligases substrate specificity governed by E3 subcellular localization (Hickey et al., 2021).

Conserved constraints governing degron potency

Considering the high conservation of QCAP pathways in the evolution of all eukaryotes, we hypothesized that QCAP degron properties are similarly well preserved. To test this paradigm, we next investigated how well the yeast-based QCDPred algorithm predicts the presence of QCAP

degrons in other organisms and selected the influenza C virus p42 protein and human serum and glucocorticoid-inducible kinase 1 (SGK1) as test cases. The influenza p42 contains a signal peptidase site at residue 259 that upon cleavage yields the p31 and CM2 proteins (**Figure 6A, *upper panel***). CM2 integrates into the ER membrane through a single TMD, while p31 is rapidly degraded by the UPS via a degron at the C-terminal region (Pekosz and Lamb, 2000). As anticipated, both the C-terminal region of p31 and the TMD of p42 were predicted by QCDPred to function as degrons (**Figure 6A, *lower panel***), however, during viral infection only the C-terminal region of p31 likely functions as a degron because it is accessible to the degradation system. Furthermore, Arteaga and co-workers have previously demonstrated that an amphipathic helix at the N-terminus of SGK1 targets the protein for proteolysis (Arteaga et al., 2006). Indeed, QCDPred analysis of SGK1 revealed three potential degrons, the strongest of which is placed between amino acids 17 and 29, the same region that was previously identified as a QCAP degron (Arteaga et al., 2006) (**Figure 6B**).

Having demonstrated that the tested non-yeast QCAP degrons can be predicted by QCDPred, we next wanted to investigate the universality of QCAP degrons by testing whether the principles established for yeast degrons generally apply to the human degronome. To this end, we assessed the correlation between PSI values, previously assigned for C-terminal regions of the entire human proteome (Koren et al., 2018), and their average QCDPred probabilities. The comparison yielded a high correlation ($\rho = -0.74$) (**Figure 6C**), similar to that observed for the yeast N-terminome ($\rho = -0.78$) (Kats et al., 2018)(**Figure 6D**), indicating that indeed principles of QCAP degron features are evolutionarily conserved. Consequently, average QCDPred scores were assigned to each amino acid in both the yeast and human proteomes (**Supplemental Tables 4,5**) and we provide QCDPred

as a general tool to predict degrons in proteins present in the UniProt database (Johansson et al., 2022).

Discussion

The discovery that E3 ligases selectively bind substrates through recognition of distinct determinants (degrons) established their role as substrate recognition modules of the UPS (Hershko et al., 1986). Consequently, the identification of degrons has become one of the focal points of the UPS research. Initially, the hunt for degrons identified mostly acquired determinants that are, for example, the result of transient post-translational modifications such as phosphorylation that induce timely, regulated degradation via dedicated E3 ligases (Zheng and Shabek, 2017). Obviously, acquired degrons do not account for the majority of QCAP, carried out by specialized E3 ligase systems that monitor protein folding state, presumably recognizing internal sequences that may become exposed following conformational aberrations. This assertion was confirmed during the characterization of *Deg1* degron of the Doa10 E3 ligase (Hochstrasser and Varshavsky, 1990; Swanson et al., 2001). *Deg1* is masked in the Mata1/Mat α2 mating-type dimer and exposed upon complex dissociation (Johnson et al., 1998). Critical for *Deg1* degron function is an amphipathic helix (Johnson et al., 1998), also present in other synthetic and authentic Doa10 substrates (Arteaga et al., 2006; Furth et al., 2011; Gilon et al., 2000; Sadis et al., 1995). Despite hydrophobicity constituting a primary Doa10-recognition determinant (Hickey et al., 2021), not all QCAP degrons conform to the same consensus (Maurer et al., 2016). Overall, the initial degron discoveries, while gaining important insights into degron complexities, could not account for the entire QCAP.

A major barrier to unearthing QCAP degrons has been their unconformity. Therefore, authentic degron discovery largely relied on the screening of peptide libraries in search of sequences that

induce the degradation of otherwise stable proteins and then, trying to deduce consensus sequence requirements (Fredrickson et al., 2011; Geffen et al., 2016; Gilon et al., 1998; Kats et al., 2018; Koren et al., 2018; Lin et al., 2018; Maurer et al., 2016; Rosenbaum et al., 2011; Sadis et al., 1995). Indeed, these efforts led to the discovery of many artificial and physiological degrons but were still far from distinguishing common degradation motifs in QCAP. A breakthrough in degron discovery has been provided by the development of the pioneering GPS-peptidome methods to study protein degradation and identify degron motifs (Koren et al., 2018; Lin et al., 2018; Tokheim et al., 2021). By implementing GPS-P in yeast we have here identified a large cohort of authentic degrons that was subsequently used to train a degron prediction algorithm (Johansson et al., 2022). Thus, this large group of peptides that determine a variety of half-lives, provide consensus degron features that are compatible with TMD properties. Further functional validation established TM-like degron features as key determinants of QCAP and possibly of the proteome half-life.

That TMDs can operate as degrons is not surprising as membrane-embedded sequences within integral ER-membrane proteins have already been shown to display a degron function. These include the C-terminal TMD of the T-cell receptor α subunit (TCR α) (Bonifacino et al., 1990; Yu et al., 1997) as well as other lone proteins that are normally part of TM protein complexes (Habeck et al., 2015; Natarajan et al., 2020). Single and homomeric ER-embedded proteins, such as the E2 enzyme Ubc6 and the C-terminal TMDs of yeast and human HMG CoA reductase can similarly undergo QCAP via their TMDs that act as degrons (Gardner and Hampton, 1999; Ravid et al., 2000; Walter et al., 2001). By demonstrating that single-pass TM proteins devoid of their SPs were rapidly degraded by the UPS (**Figures 4E, 4F**), we have expanded this view by establishing TMDs as conserved QCAP degrons of non-integrated TM proteins.

Our findings that TMDs can act as degrons are compatible with a pre-insertion degradation mechanism, operating at the cytosolic side of the ER membrane, that subjects Signal Recognition Particle (SRP)-independent substrates of the glycosylphosphatidylinositol (GPI) anchored proteins to QCAP (Ast et al., 2014). It is also compatible with SRP-independent insertion mechanisms of Atg27 and Ksh1 (Costa et al., 2018) that eliminate subpopulations that may evade the secretory pathway (**Figure 4F**). Moreover, in mammals, BAG6 and its associated protein UBQLN4 were shown to recognize the exposed hydrophobicity of TMDs of proteins that evade the secretory pathway and trigger their proteasomal degradation (Hessa et al., 2011; Suzuki and Kawahara, 2016). Thus, exposure of both the SP and TMDs may ensure that suboptimal or complete failure of ER membrane integration would result in QCAP. Failure to degrade mislocalized membrane proteins may result in cytotoxicity, due to enhanced formation of insoluble intracellular bodies or aberrant insertion in the mitochondria membrane (Costa et al., 2018). Our observation that SP-devoid integral membrane proteins are subjected to rapid degradation (**Figures 4E-F**) is fully compatible with this assumption.

The prevalence of QCAP degrons function was previously demonstrated by overlapping degron recognition by E3 ligases **(Figure 5G** and (Breckel and Hochstrasser, 2021)). As protein structure is dynamic, QCAP sensing of protein folding state via the exposure of TM-like domains and their nonselective E3 ligase recognition can now provide a plausible mechanism for regulation of the proteome stability. As E3 ligases are rate-limiting for ubiquitin-dependent degradation, recognition of common QCAP degron features by multiple E3 ligases may increase cellular degradation capacity in response to diverse stress conditions where aberrant protein overload might lead to proteotoxicity (Buchberger et al., 2010).

Molecular chaperones that discern misfolded proteins also participate in misfolded protein degradation (Kriegenburg et al., 2012; Shiber and Ravid, 2014). Auxiliary PQC chaperone functions include substrate solubilization, mediating E3 binding, as well as delivery of ubiquitinated proteins to the proteasome (Shiber and Ravid, 2014). The prevailing mode of chaperones' function in QCAP asserts that Hsp70s initially recognize misfolded substrates and deliver them to an E3 ligase, a function facilitated by Hsp40s and nuclear exchange factors that catalyze ATP hydrolysis and ADP exchange, respectively (Abildgaard et al., 2020). While a role for Hsp70s/Hsp40s in misfolded substrates targeting the human E3 ligase, the carboxy-terminus of Hsc70 interacting protein (CHIP), is well established (McDonough and Patterson, 2003), whether Hsp70s/Hsp40s similarly mediate the recognition of QCAP degrons by their cognate E3s remains to be determined.

Conclusion

Adaptation of the GPS-P technology into yeast research facilitated the identification of QCAP degron sequences within the eukaryotic peptidome. The substantial number of identified degron sequences thus enabled a machine-learning algorithm to faithfully define consensus degron features. The ability of QCDPred to identify degrons in viral and human proteins (**Figure 6**), supports the notion that the majority of the proteome contains QCAP degrons with conserved characteristics.

The extent of TM-like degron function remains to be determined. Whether it is the "Holy Grail" that controls proteome stability or if other degron modules also play a substantial role remains to be elucidated. However, the properties of TM-like domains and their conditional exposure can now, for the first time, provide an expansive explanation of QCAP function in the maintenance of the eukaryotic proteome.

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Author contributions

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Declaration of Interests

The authors declare no competing interests.

Figure titles and legends

Figure 1. Principles and validation of the yGPS-peptidome method.

(A) *Upper panel:* Schematic representation of yGPS-peptide library (yGPS-P_{lib}) where a peptide-DNA library comprised of 51mer tiled DNA fragments from 355 proteins, represented by different colors, are cloned in-frame downstream to GFP in the yGPS-P vector. The single mRNA product consists of yeCherry-IRES-yeGFP-peptide. *Lower panel*: DNA and protein sequences illustrating the cloning site that pursues GFP and the pentameric linker.

(B) Flowchart of the degron screen. yGPS-P_{lib} is transformed into yeast, followed by flow cytometry or FACS.

(C) *Upper panel*: Flow cytometry histograms of *CL1* or *DegAB* degrons appended to yGPS-P, in *wild type* and *doa10*Δ cells. Fluorescence emissions of 10,000 cells were determined for each condition. *Lower panel*: Immunoblot analysis of the levels of *CL1*- and *DegAB*- appended GFP compared to Cherry loading control. yeG: yeGFP, yeC: yeCherry, EV: empty vector.

(D) Flow cytometry histogram of yeG/yeC in yGPS-P_{lib} compared to empty vector control.

(E) Proteasome dependence of GFP-appended peptides. Cells expressing yGPS-P_{lib} were treated with 10µM Bortezomib (BZ) for four hours or with DMSO vehicle control. Cells were subjected to immunoblotting with anti-ubiquitin Abs (left panel), or to flow cytometry analysis (middle panel). Shown in the right panel is the effect of BZ on a pre-selected degron library yGPS-P_{deg} composed of top10% degrons.

Figure 2. QCAP degrons functionality is largely defined by their amino acid composition.

(A) Fluorescence-activated cell sorting of yGPS-P_{lib} into four gates (G1-4), each composed of 2.5 million cells, was followed by NGS and protein stability index (PSI) scoring of each peptide, leading to the formation of a peptide density map. Shown is a FACS illustration and gating of 10,000 cells. Degron cutoff at PSI < 1.7 is marked by a dashed line the

(B) Validation of QCDPred-based degron predictions. Ten library peptides were re-cloned into yGPS-P, followed by flow cytometry analysis. The peptides were divided into three different groups: Group I- PSI ≤ 1.62, P ≥ 0.85; Group II- PSI > 1.62, P < 0.85; Group III- PSI ≤ 1.62, P < 0.85. EV: empty vector.

(C) Scatter plot comparing between Kyle-Doolittle hydrophobicity amino acid scores and QCDPred probabilities.

(D) Flow cytometry histogram of intact or mutant P3 peptide in which three amino acids were replaced with either glutamate (E) or arginine (R) residues.

(E) 3D structure of QCDPred-calculated cytosolic Pca1 degron (amino acids 289-305), based on AlphaFold Pca1 structural model #AF-p38360-F1 (Conf. (90 > pLDDT > 70). Marked by a blue color are the cysteine residues. Marked by a red color are the three amino acids that have been replaced with aspartate or arginine. pLDDT: per-residue confidence score on a scale of 0-100.

(F) Immunoblot analysis of Pca1 protein levels. *Wild type* or *doa10*Δ (*d*Δ) cells, expressing the indicated HA-tagged Pca1 proteins were left intact or treated with 50 μM $CdCl_2$ for 1 hour. To determine degradation dynamics, where indicated, the translation inhibitor cycloheximide (CHX) was added to cells for 15 min prior to cell harvesting. Pca1 levels were measured by immunoblotting using anti-HA Abs while Pgk1 levels served as a loading control. D: replacement of residues marked in 2F by red color with aspartate; R: replacement of residues marked in 2F by red color with Arginine.

Figure 3. QCDPred assignment of TMDs as QCAP degrons.

(A) Map of degrons prediction by QCDPred (in grey) and TMDs prediction by TMHMM (in light blue) within Pca1 sequence. Marked by an arrowhead is the Pca1 cytosolic degron. A degron cutoff probability is marked at P = 0.85 by a dashed red line.

(B) Venn diagram of the relationship between yeast TMDs, extracted from the TM Helix Hidden Markov Model (TMHMM) algorithm, and QCDPred degron probabilities (P ≥ 0.85).

(C) Assignment of QCDPred calculated degrons (in green) and TMHMM predicted TMDs (in yellow) to the cytochrome b-c1 complex (PDB #6T0B). overlay regions are colored in orange.

(D) Frequency distribution of (i) all QCDPred assigned degrons ($P \geq 0.85$), (ii) degrons composed of TMDs, and (iii) degrons that are not part of a TMD, sorted into probability ranges between 0.85 -1.

Figure 4. Transmembrane domains function as QCAP degrons.

(A) Membrane localization of single-pass type-1 TM proteins, Erp2, Atg27, and Ksh1, determined by fluorescence microscopy.

(B) Map of QCDPred and TMHMM predictions of Erp2, Atg27, and Ksh1 degron and TMD probabilities, respectively. A degron cutoff probability is marked at $P = 0.85$ by a dashed red line.

(C, D, E, F). Flow cytometry histograms of the yeG/yeC ratio in cells expressing (C, D) C-terminal appended TMDs (C) or SPs (D), (E) N-terminal appended, full length and ΔSP proteins, and (F) C-terminal appended ΔSP proteins. Illustrated below each panel are the composition and domain order of the GFP-appended proteins. TM: transmembrane, SP: signal peptide.

Figure 5. Characterization of QCAP degrons.

(A) Comparison of mean amino acid distribution between TMDs with high degron probability ($P \geq 0.85$, N=6,064) to those with lower probability ($P < 0.85$, N=107). Error bars represent low and high confidence intervals.

(B) Pie chart of the classification and relative proportions of protein secondary structure within the yeast proteome versus that of QCDPred-predicted degrons, based on the AlphaFold Protein Structure Database.

(C) Plot of the percentile of α helix structures versus QCDPred assigned non-TMD degron probabilities divided into four equal bins.

(D) Flow cytometry histograms of the yeG/yeC ratio in cells expressing P1 peptide emerged from the yeast protein COB and two randomly scrambled variants SC1 and SC2.

(E) Plot of QCDPred calculated degron centers, as a function of protein length. ρ: Spearman's correlation coefficient. $P_{value} < 0.0001$.

(F) Gene ontology (GO) process annotations of the fold change of top twelve significant pathways (P-value < 0.05) enriched or devoid of degrons, compared to a reference yeast proteome (strain S288C).

(G). Venn diagram displaying overlapping functions of the tested QCAP E3 enzymes. ΔPSI values between degrons in *pdr5*Δ strain (PSI < 1.7, 2175 peptides) *and E3*Δ strains were calculated. Sequences of top 10% ΔPSI values for each knockout strain (218 peptides) were compared.

Figure 6. Degron projections in non-yeast organisms

(A) *upper panel* Assignment of QCDPred projected degrons into the influenza C virus Polyprotein p42 trRosetta-predicted 3D structure (Template modeling score: 0.642). Shown are the schematics of p42 cleavage at an internal SP site (marked in purple) into p31 and CM2. *Lower panel*: QCDPred and TMHMM probabilities of p42 degrons (in grey) and TMDs (in light blue), respectively. *Arrowhead* indicates the position of the postulated p31 degron. `A degron cutoff probability is marked at P = 0.85 by a dashed red line.

(B) *Upper panel*: Assignment of a QCDPred-identified QCAP degron into the AlphFold-predicted SGK1 structure (#AF-O00141-F1). *Lower panel*: QCDPred-calculated degron probabilities within

SGK1. *Arrowhead* indicates the position of the postulated SGK1 degron. A degron cutoff probability is marked at P = 0.85 by a dashed red line.

(C) Scatter plot comparing PSI values of 23mer C-terminal peptides of the human proteome (Koren et al., 2018) and averaged QCDPred assessment of the same fragments. Prediction scores were averaged between 7 different amino acid centers within the 23 amino acids length of the original peptides. Shown are values of random 25% peptides from Koren's screen. ρ: Spearman's correlation coefficient. $P_{value} < 0.0001$.

(D) Scatter plot comparing PSI values of 15mer N-terminal peptides of the yeast *Saccharomyces cerevisiae* proteome (Kats et al., 2018) and QCDPred assessment of the same fragments. ρ: Spearman's correlation coefficient. $P_{value} < 0.0001$.

Materials and Methods

Parental plasmid for yGPS-P screen

Plasmid pGADT7-ADH700-yeCherry-p150-yeGFP-DHFR was obtained from Addgene (#24378) (Edwards and Wandless, 2010) and was used as a template for PCR cloning of ADH700-yeCherry-P150-yeGFP into pTR1412 (Geffen et al., 2016) at NotI and XmaI restriction sites. A 5-mer linker was added downstream to GFP to create the parental plasmid pTR1861 for yGPS-P screen (Figure 1A). pTR2089, the parental plasmid for yGPS-P N-terminal cloning, was constructed by overlap extension PCR (Hilgarth and Lanigan, 2020), producing a fragment containing PacI and BamHI restriction sites, that was placed upstream to GFP in pTR1861 by ligation.

Cloning and mutagenesis

Plasmids used in this study are listed below. DNA fragments encoding the full-length, ΔSP, TMD only, and SP only versions of Erp2, Atg27, and Ksh1 were amplified from genomic DNA of *wild-type* yeast strain BY4741. The PCR fragments were subjected to digestion with restriction enzymes XmaI and NheI (for C-terminal cloning) or with PacI and BamHI (for N-terminal cloning). The resulting fragments were subcloned into pTR1861 or pTR2089, respectively.

Oligos insertions into pTR1861 were done by heating a single DNA pair containing the wanted insertion (see "Oligos Table" for details) and flanking sequences responsible for overhangs compatible with XmaI and NheI restriction sites and cooling down slowly to create double-stranded fragments. The double-strand DNA was ligated directly into pTR1861, digested by the same restriction enzymes. Mutagenesis of peptide P3 and of Pca1 was conducted using QuikChange Lightning Site-Directed Mutagenesis Kit, according to the manufacturer's instructions (Agilent). All products were verified by sequencing.

Generation of a peptide library (yGPS-P_{lib})

Three hundred thirty-five proteins, corresponding to 23 yeast complexes were first encoded as DNA bases using the Saccharomyces Genome Database (SGD) website. Then, DNA corresponding to the open reading frame of each protein was divided into 51 bp (17-mer) fragments with 36 bp (12-mer) overlaps between neighboring oligonucleotides (tiling). The fragments also contained two flanking 12 bp primers that match the vector sequence to enable Gibson assembly. The corresponding oligonucleotides were synthesized by LC Sciences (Houston, TX), amplified by PCR, and cloned by Gibson assembly master mix kit (New England Biolabs) into pTR1861 at XmaI and NheI restriction sites, followed by transformation into electro-competent DH10B bacterial cells. Approximately two million colonies were scraped from plates and pooled and plasmid DNA was purified using PureLink® HiPure plasmid filter Midiprep kit (Invitrogen). The

resulting plasmid library was transformed into TRy1392 *pdr5*Δ yeast strain, followed by selection on leucine-deficient media (SD-Leu). Surviving cells were scraped, pooled, and frozen immediately in 25% glycerol at -80°C.

Generation of a degron library (yGPS-P_{deg})

Yeast cells expressing yGPS-P_{lib} were grown O/N on SD-Leu media to mid-log phase. Cells were subjected to BD FACS Aria III instrument using 488 nm and 561 nm lasers for capturing the fluorescence emission of GFP and Cherry, respectively. One million cells having the lowest 10% yeG/yeC ratio, representing cells harboring unstable GFP, were separated (Supplemental Figure 1). Sorted cells were incubated O/N in SD-Leu media, then divided into aliquots and frozen in 25% glycerol at -80°C.

Degron screen

Cells expressing yGPS-P_{lib} were grown to mid-log-phase and sorted by FACS BD-Facsaria III into four equal gates, each containing 2.5 million yeast cells, based on their yeG/yeC ratio (Figure 2A). Plasmids from each gate were purified using Zymoprep yeast Plasmid Miniprep II (Zymo Research).

Preparation of plasmid DNA for NGS

DNA sequencing by NGS consisted of two PCR amplification steps with KAPA HiFi HotStart ReadyMix PCR Kit (Roche). The first step (18 cycles) was performed using primers flanking the peptides, with overhangs complementary to Illumina adapters (primers NGS-F, NGS-R). The second step was performed using standard N-series Illumina barcoded adapters (12 cycles). Sequences were size selected using SPRI beads for NGS. Samples were subsequently pooled, purified on an agarose gel, and sequenced on an Illumina NextSeq 500 machine.

Data pre-processing

Sequencing data were processed using a custom pipeline written for the R project for statistical computing (https://www.R-project.org). Reads were aligned to the expected oligo database with bowtie2 (Langmead and Salzberg, 2012). Sequences corresponding to the tiled peptides were counted and assigned to the different strains based on forward and reverse barcodes.

Protein stability indices (PSIs) were calculated according to Yen et al (Yen et al., 2008). Briefly, the frequency, f_{ig}, of peptide, i, in gate, g, was multiplied by gate number (1-4) and summed up, yielding a stability score between 1 (maximally unstable) and 4 (maximally unstable)

$$PSI_i = \frac{\sum_g g \times f_{ig}}{\sum_g f_{ig}}$$

Flow cytometry

Yeast cells were grown to mid-log-phase on SD-Leu media and analyzed on a CellStream analyzer instrument (Merck) using 488 nm and 561 nm lasers for capturing the fluorescence emission of GFP and Cherry, respectively. For each condition, 10,000 events were analyzed and presented on histogram using FlowJo software (BD Biosciences). Experiments were repeated two or more times.

Immunoblotting

Cells were grown to mid-log-phase and pelleted by centrifugation (3,500 × g, 5 min). Cells were incubated with 0.1N NaOH for 5 min, then pelleted by centrifugation (17,000 × g, 3 min) and SDS-PAGE sample buffer containing 50 mg/ml Dithiothreitol was added, followed by boiling for 5 min. Proteins were separated on SDS-PAGE, transferred to a PVDF membrane, blocked in 10% Dry Milk in TBS + 0.1% Tween-20 (TBS-T), and then probed with primary antibodies for one hour at room temperature. Following three washes with TBS-T, the membrane was incubated with a secondary antibody for 0.5 h at room temperature, then washed three times with TBS-T.

Membranes were incubated with ECL mix (Thermo Fisher Scientific) for 2 min and reactive bands were visualized using Fusion Pulse (Vilber Lourmat).

Fluorescence Microscopy:

Imaging was performed with Olympus IX71 inverted microscope with an x 60 oil objective lens. Fluorescence was excited with 576 nm for Cherry and with 488 nm for GFP. Imaging data were handled using ImageJ.

Proteome Databases

All proteome databases were downloaded from the UniProt database server (https://www.uniprot.org) as FASTA files. These files contain the full-length protein sequence.

PDB/CIF data

AlphaFold-2 based PDB and CIF files for single proteins were extracted from the European Bioinformatics Institute website (https://alphafold.ebi.ac.uk/). These files were used for creating a 3D proteins database. Most PDB and CIF files contain information on secondary structure patterns. Each amino acid in these models was assigned secondary structure indicator and proteome statistics were inferred.

Transmembrane protein Data

Transmembrane protein data was extracted from the TM Helix Hidden Markov Model (TMHMM) algorithm (Krogh et al., 2001; Tusnády and Simon, 1998), implemented with python package tmhmm.py. To compute the intersection between TMDs and degrons, each amino acid within the entire yeast proteome was evaluated for QCDPred value and TMD classification. Amino acids with QCDPred probability ≥ 0.85 that were classified as TMDs according to TMHMM were added to the intersection group.

Antibodies used in this study:

Polyclonal Rabbit anti-GFP	Abcam	ab290
Monoclonal Rabbit anti-mCherry	Abcam	ab213511
Monoclonal Rat anti-HA	Roche	11867423001
Monoclonal Mouse anti-PGK1	Abcam	ab113687
Polyclonal Rabbit anti-G6PD	Sigma Israel	a9521
Polyclonal Rabbit anti-Ubiquitin	Dako	Z0458
Goat anti-Rabbit	ImmunoResearch	111035144
Goat anti-Mouse	ImmunoResearch	115035062
Goat anti-Rat	ImmunoResearch	112035062

Chemicals:

Chemical	Manufacturer	Catalog #
Bortezomib (BZ)	A2S	A2614
Cadmium	Sigma	265365

Yeast strains used in this study:

Strain name	Genotype
BY4741	*MATa met15Δ0, ura3Δ0, leu2Δ0, his3Δ1*
TRy1121	*MATa met15Δ0, ura3Δ0, leu2Δ0, his3Δ1, doa10::KanMX*
TRy1373	*MATa met15Δ0, ura3Δ0, leu2Δ0, his3Δ1, ubr1::KanMX*
TRy1388	*MATa met15Δ0, ura3Δ0, leu2Δ0, his3Δ1, san1::KanMX*
TRy1392	*MATa met15Δ0, ura3Δ0, leu2Δ0, his3Δ1, pdr5::KanMX*

Oligos Table:

name	sequence
NGS-F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACANNNGGATCAGCTGGCTCACCC
NGS-R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGANNNACATAACTAATTACATGATCAGTCAGC
P1_F	CCGGGTATTTAGTACCTTTTATCATTGCTGCAATGGTTATTATGCATTTAATGGCATGAG
P1_R	CTAGCTCATGCCATTAAATGCATAATAACCATTGCAGCAATGATAAAAGGTACTAAATAC
P2_F	CCGGGATCATGAGGTGCAAAATATATTTCTTTTACTTAAGGATATCAAAAAAGCTATGAG
P2_R	CTAGCTCATAGCTTTTTTGATATCCTTAAGTAAAAGAAATATATTTTGCACCTCATGATC

P3_F	CCGGGTTCAAACGAAACGCTGTTTTTGTTGGTACTATCTTTGCAGGTGCCTTTGTTTGAG
P3_R	CTAGCTCAAACAAAGGCACCTGCAAAGATAGTACCAACAAAAACAGCGTTTCGTTTGAAC
P4_F	CCGGGAACAAGTTACACTACTGTGTTTCTTGTGCTATTCACGCCAGAATTGTCAGATGAG
P4_R	CTAGCTCATCTGACAATTCTGGCGTGAATAGCACAAGAAACACAGTAGTGTAACTTGTTC
P5_F	CCGGGTCTTTGTATTTGCTATCTATCTGGGTGAAGAAGTTCAAATGGGCCGGTATCTGAG
P5_R	CTAGCTCAGATACCGGCCCATTTGAACTTCTTCACCCAGATAGATAGCAAATACAAAGAC
P6_F	CCGGGGACGAACCTGATGAACAAGGTAACCCTAAAAAGAGACCAGGTAAGTTGTCCTGAG
P6_R	CTAGCTCAGGACAACTTACCTGGTCTCTTTTTAGGGTTACCTTGTTCATCAGGTTCGTCC
P7_F	CCGGGTCGGGTAAAAGTAACACCTCAAACGATGCTTCTAACAAAAAAGAAACCAAGTGAG
P7_R	CTAGCTCACTTGGTTTCTTTTTTGTTAGAAGCATCGTTTGAGGTGTTACTTTTACCCGAC
P8_F	CCGGGGTTATAGATAAACTAAAAAATGAAAACACGGATTTGAAAGACATTATCCAATGAG
P8_R	CTAGCTCATTGGATAATGTCTTTCAAATCCGTGTTTTCATTTTTTAGTTTATCTATAACC
P9_F	CCGGGTTAAATATCAGAATTCAAACCTACGGTGAAACGACCGCGGTGGATGCTCTTTGAG
P9_R	CTAGCTCAAAGAGCATCCACCGCGGTCGTTTCACCGTAGGTTTGAATTCTGATATTTAAC
P10_F	CCGGGAAGGTTGGGTCTATACGGGTTCACAAATCTGGAAAACTTTCCGTGAAAATTTGAG
P10_R	CTAGCTCAAATTTTCACGGAAAGTTTTCCAGATTTGTGAACCCGTATAGACCCAACCTTC
P3 E_{mut}_F	CCGGGTTCAAACGAAACGCTGTTTTTGTTGGTACTGAGGAGGCAGGTGCCGAGGTTTGAG
P3 E_{mut}_R	CTAGCTCAAACCTCGGCACCTGCCTCCTCAGTACCAACAAAAACAGCGTTTCGTTTGAAC
P3 R_{mut}_F	CCGGGTTCAAACGAAACGCTGTTTTTGTTGGTACTCGTCGTGCAGGTGCCCGTGTTTGAG
P3 R_{mut}_R	CTAGCTCAAACACGGGCACCTGCACGACGAGTACCAACAAAAACAGCGTTTCGTTTGAAC
Scr1_F	CCGGGATCTTATTTATGGTAGCTATTCCTGTTCATGCAATTGCATATTTAATGATGTGAG
Scr1_R	CTAGCTCACATCATTAAATATGCAATTGCATGAACAGGAATAGCTACCATAAATAAGATC
Scr2_F	CCGGGCATGCTGCAATCATGTATATGGCAGTTATTGTATTAATTTTTTTAATGCCTTGAG
Scr2_R	CTAGCTCAAGGCATTAAAAAAATTAATACAATAACTGCCATATACATGATTGCAGCATGC

<u>List of plasmids used in this study</u>

Unless indicated otherwise, clones are C-terminal fusions to yeGFP

	Description	Additional information
pTR1494	pRS416-GPDp-HA-Pca1	adle et al. PNAS 2009
pTR1859	pRS315-ADH700p-yeCherry-p150-yeGFP-XmaI,NheI-CYCt	
pTR1861	pRS315-ADH700p-yeCherry-p150-yeGFP-linker-XmaI,NheI-CYCt	Vector for C-terminal fusion to yeG
pTR1882	pRS315-ADH700p-yeCherry-p150-yeGFP-linker-XmaI-*CL1*-NheI-CYCt	
pTR1884	pRS315-ADH700p-yeCherry-p150-yeGFP-linker-XmaI-*DegAB*-NheI-CYCt	
pTR2036	pRS315-ADH700p-yeCherry-p150-yeGFP-linker-XmaI-P1-NheI-CYCt	
pTR2037	pRS315-ADH700p-yeCherry-p150-yeGFP-linker-XmaI-P2-NheI-CYCt	
pTR2038	pRS315-ADH700p-yeCherry-p150-yeGFP-linker-XmaI-P3-NheI-CYCt	
pTR2058	pRS315-ADH700p-yeCherry-p150-yeGFP-linker-XmaI-$P3_{EE}$-NheI-CYCt	Replacing TIFAGAFV with TEEAGAEV
pTR2060	pRS315-ADH700p-yeCherry-p150-yeGFP-linker-XmaI-$P3_{RR}$-NheI-CYCt	Replacing TIFAGAFV with TRRAGARV
pTR2039	pRS315-ADH700p-yeCherry-p150-yeGFP-linker-XmaI-P4-NheI-CYCt	
pTR2040	pRS315-ADH700p-yeCherry-p150-yeGFP-linker-XmaI-P5-NheI-CYCt	
pTR2041	pRS315-ADH700p-yeCherry-p150-yeGFP-linker-XmaI-P6-NheI-CYCt	

pTR2042	pRS315-ADH700p-yeCherry-p150-yeGFP-linker-XmaI-P7-NheI-CYCt	
pTR2043	pRS315-ADH700p-yeCherry-p150-yeGFP-linker-XmaI-P8-NheI-CYCt	
pTR2044	pRS315-ADH700p-yeCherry-p150-yeGFP-linker-XmaI-P9-NheI-CYCt	
pTR2045	pRS315-ADH700p-yeCherry-p150-yeGFP-linker-XmaI-P10-NheI-CYCt	
pTR2089	pRS315-ADH700p-yeCherry-p150-PacI,BamHI-yeGFP-linker-XmaI,NheI-CYCt	Vector for N-terminal fusion to yeG
pTR2124	pRS416-GPDp-HA-Pca1$_{DD}$	Replacing LGCICNY with LDCDCDY
pTR2127	pRS315-ADH700p-yeCherry-p150-yeGFP-linker-XmaI-$_{\Delta sp}$Erp2-NheI-CYCt	
pTR2128	pRS315-ADH700p-yeCherry-p150-yeGFP-linker-XmaI-$_{\Delta sp}$Atg27-NheI-CYCt	
pTR2129	pRS315-ADH700p-yeCherry-p150-yeGFP-linker-XmaI-$_{\Delta sp}$Ksh1-NheI-CYCt	
pTR2149	pRS315-ADH700p-yeCherry-p150-PacI-Erp2-BamHI-yeGFP-linker-CYCt	N-terminal fusion
pTR2151	pRS315-ADH700p-yeCherry-p150-PacI-Atg27-BamHI-yeGFP-linker-CYCt	N-terminal fusion
pTR2152	pRS315-ADH700p-yeCherry-p150-PacI-Ksh1-BamHI-yeGFP-linker-CYCt	N-terminal fusion
pTR2153	pRS315-ADH700p-yeCherry-p150-PacI-$_{\Delta sp}$Erp2-BamHI-yeGFP-linker-CYCt	N-terminal fusion
pTR2155	pRS315-ADH700p-yeCherry-p150-PacI-$_{\Delta sp}$Atg27-BamHI-yeGFP-linker-CYCt	N-terminal fusion
pTR2156	pRS315-ADH700p-yeCherry-p150-PacI-$_{\Delta sp}$Ksh1-BamHI-yeGFP-linker-CYCt	N-terminal fusion
pTR2160	pRS315-ADH700p-yeCherry-p150-yeGFP-linker-XmaI-SP(Ksh1)-NheI-CYCt	SP of Ksh1 (aa 1-26)
pTR2162	pRS315-ADH700-yeCherry-p150-yeGFP-linker-XmaI-SP(Erp2)-NheI-CYCt	SP of Erp2 (aa 1-26)
pTR2164	pRS315-ADH700p-yeCherry-p150-yeGFP-linker-XmaI-SP(Atg27)-NheI-CYCt	SP of Atg27 (aa 1-19)
pTR2167	pRS416 GPD HA-Pca1$_{RR}$	replacing LGCICNY with LRCRCRY
pTR2171	pRS315-ADH700p-yeCherry-p150-yeGFP-linker-XmaI-SC1-NheI-CYCt	Scrambled P1, variant #1
pTR2172	pRS315-ADH700p-yeCherry-p150-yeGFP-linker-XmaI-SC2-NheI-CYCt	Scrambled P1, variant #2
pTR2213	pRS315-ADH700p-yeCherry-p150-yeGFP-linker-XmaI-TMD(Atg27)-NheI-CYCt	TMD of Atg27 (aa 200-220)
pTR2214	pRS315-ADH700p-yeCherry-p150-yeGFP-linker-XmaI-TMD(Erp2)-NheI-CYCt	TMD of Erp2 (aa 183-203)
pTR2215	pRS315-ADH700p-yeCherry-p150-yeGFP-linker-XmaI-TMD(Ksh1)-NheI-CYCt	TMD of Ksh1 (aa 54-72)

Supplemental Information titles and legends

Supplemental Figures

Supplemental Figure 1, related to Figure 1. Isolation of top 10% degrons. Yeast cells expressing yGPS-P$_{lib}$ Were grown to mid-log-phase, followed by FACS of top 10% degron population. One million cells comprising the degron library (yGPS-P$_{deg}$) were collected, then grown O/N and frozen in liquid nitrogen or taken for further analysis. Illustrated is gating of top 10% degron population out of a total of 10,000 cells.

Supplemental Figure 2. related to Figure 2. Maps of PSI values emerged from experimental data (black dots), average PSI values for each amino acid (colored in black), and calculated QCDPred

probabilities (colored in red) were plotted for each of the proteins in the screen that reach 100% coverage (N=306).

Supplemental Figure 3, related to Figure 2. Group-I and group-III peptides are substrates of the proteasome. Cells expressing the related fusion peptides were incubated with DMSO (vehicle) or with 10µM (Bortezomib) BZ for four hours. The increased yeG/yeC in group-I and group-III peptides indicate proteasome-dependent degradation. No significant effect of BZ on Group-II peptides was observed.

Supplemental Figure 4, related to figure 3. Degrons in the b-c1 complex. (A) PSI values emerged from experimental data (black dots), average PSI values for each amino acid (colored in black), QCDPred probabilities (colored in red), and TMHMM prediction (colored in light blue) were plotted for each subunit of the b-c1 complex. A degron cutoff probability is marked at P = 0.85 by a dashed grey line. (B) Assignment of QCDPred projected Degrons (green) and TMHMM predicted TMDs (yellow) to TM proteins in the b-c1 3D structure (PDB #6t0b). Overlying regions are colored in orange.

Supplemental Figure 5, related to Figure 4. Immunoblot analysis of Erp2, Atg27 and Ksh1, with or without their SPs. Where indicated, 10 µM of Bortezomib (BZ) were added to cells for 4 hrs. prior to cell harvesting. *FL*- full-length proteins. Δ*SP*- proteins devoid of the SP.

Supplemental Figure 6, related to Figure 5. Structure prediction of GFP-fused peptides using trRosseta. Estimated Template Modeling scores: COB- 0.856, SC1-0.865, SC2-0.828.

Supplemental Figure 7, related to Figure 5. Large-scale GO annotations of all significant processes (p-value < 0.05) that are either enriched or devoid of predicted degrons at P ≥ 0.85.

Supplemental Figure 8, related to figure 5. QCDPred analysis of key cytosolic/nuclear yeast chaperones. A degron cutoff probability is marked at P = 0.85 by a dashed red line.

Supplemental Tables

Supplemental Table 1, related to Figure 1. proteins in the screen and the complex they belong to.

Supplemental Table 2, related to Figure 2. PSI values, average PSI, and degron probabilities for peptides in the screen

Supplemental Table 3, related to Figure 2. List of re-evaluated peptides on the screen and their PSI and QCDPred probability scores.

Supplemental Table 4, related to Figure 6. QCDPred analysis of the yeast proteome.

Supplemental Table 5, related to Figure 6. QCDPred analysis of the human proteome.

References

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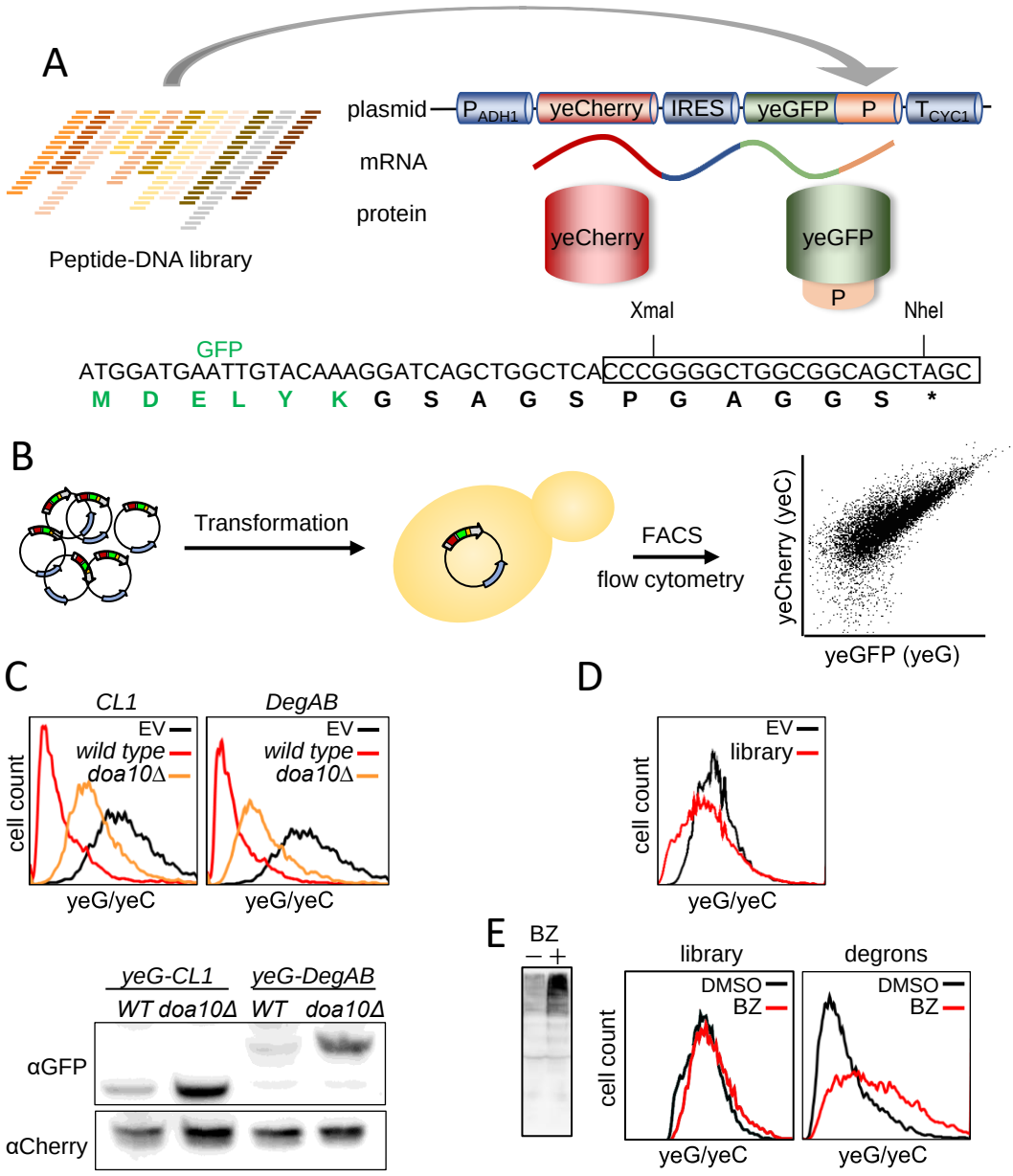
Figure 1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Peptide-DNA library
plasmid
P ADH1
yeCherry
IRES
yeGFP
P
T CYC1
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protein
yeCherry
yeGFP
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XmaI
NheI
GFP
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M D E L Y K G S A G S P G A G G S *
B
Transformation
FACS
flow cytometry
yeCherry (yeC)
yeGFP (yeG)
C
CL1
DegAB
EV
wild type
doa10Δ
cell count
yeG/yeC
yeG/yeC
yeG-CL1
yeG-DegAB
WT doa10Δ WT doa10Δ
αGFP
αCherry
D
EV
library
cell count
yeG/yeC
E
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library
degrons
DMSO
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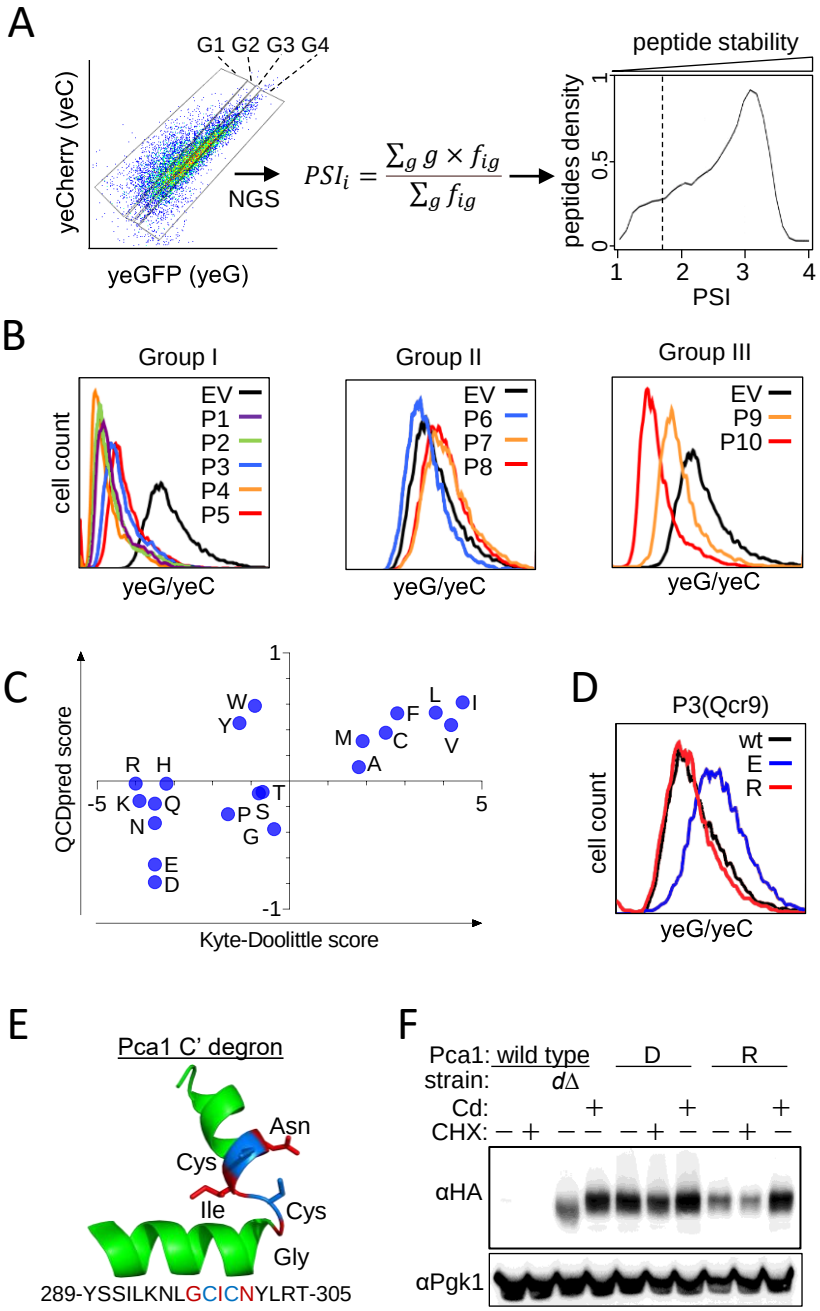
Figure 2


A
G1 G2 G3 G4
yeCherry (yeC)
yeGFP (yeG)
NGS
$PSI_i = \frac{\sum_g g \times f_{ig}}{\sum_g f_{ig}}$
peptide stability
peptides density
PSI
B
Group I
Group II
Group III
cell count
EV
P1
P2
P3
P4
P5
P6
P7
P8
P9
P10
yeG/yeC
C
QCDpred score
Kyte-Doolittle score
D
P3(Qcr9)
wt
E
R
E
Pca1 C' degron
Asn
Cys
Ile
Cys
Gly
289-YSSILKNLGCICNYLRT-305
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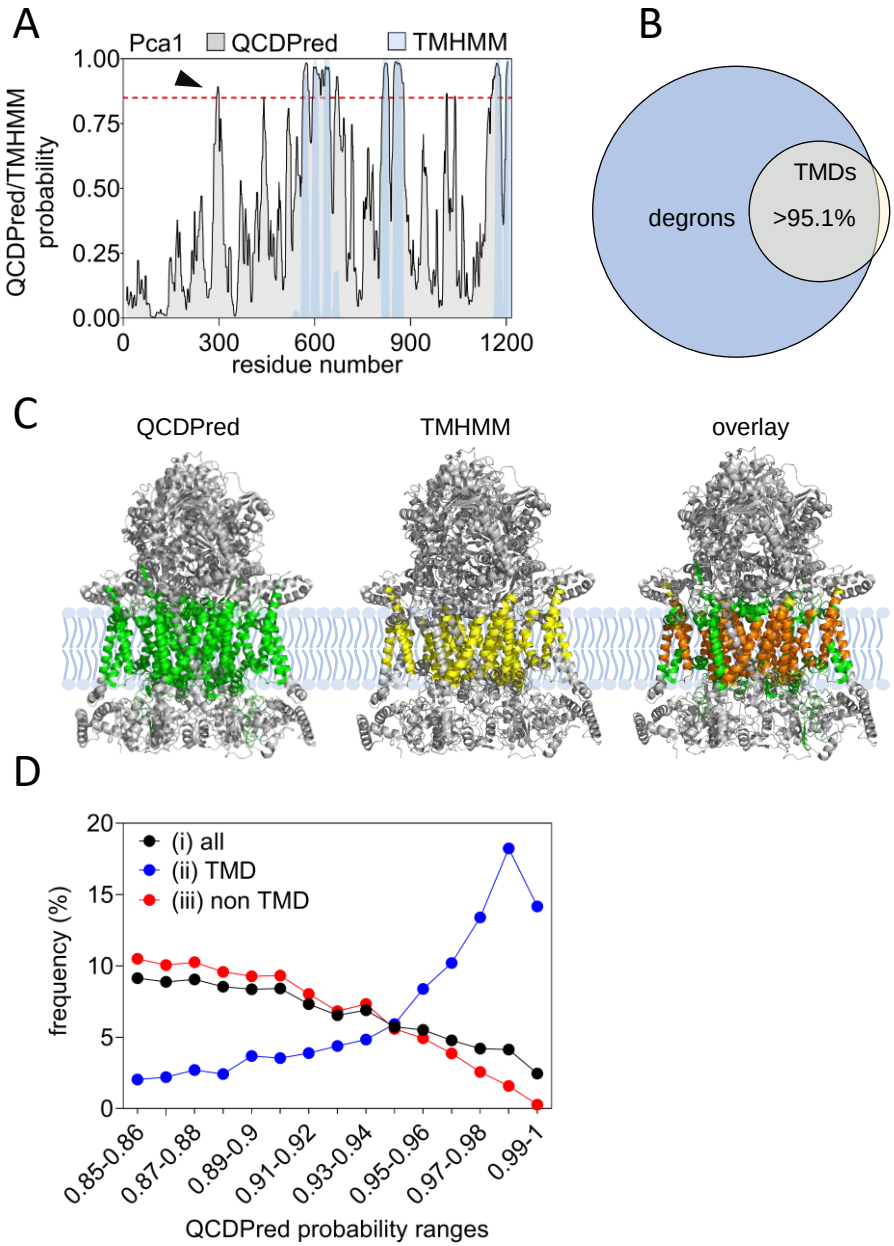
Pca1: wild type
D
R
strain:
dΔ
Cd:
CHX:
αHA
αPgk1

Figure 3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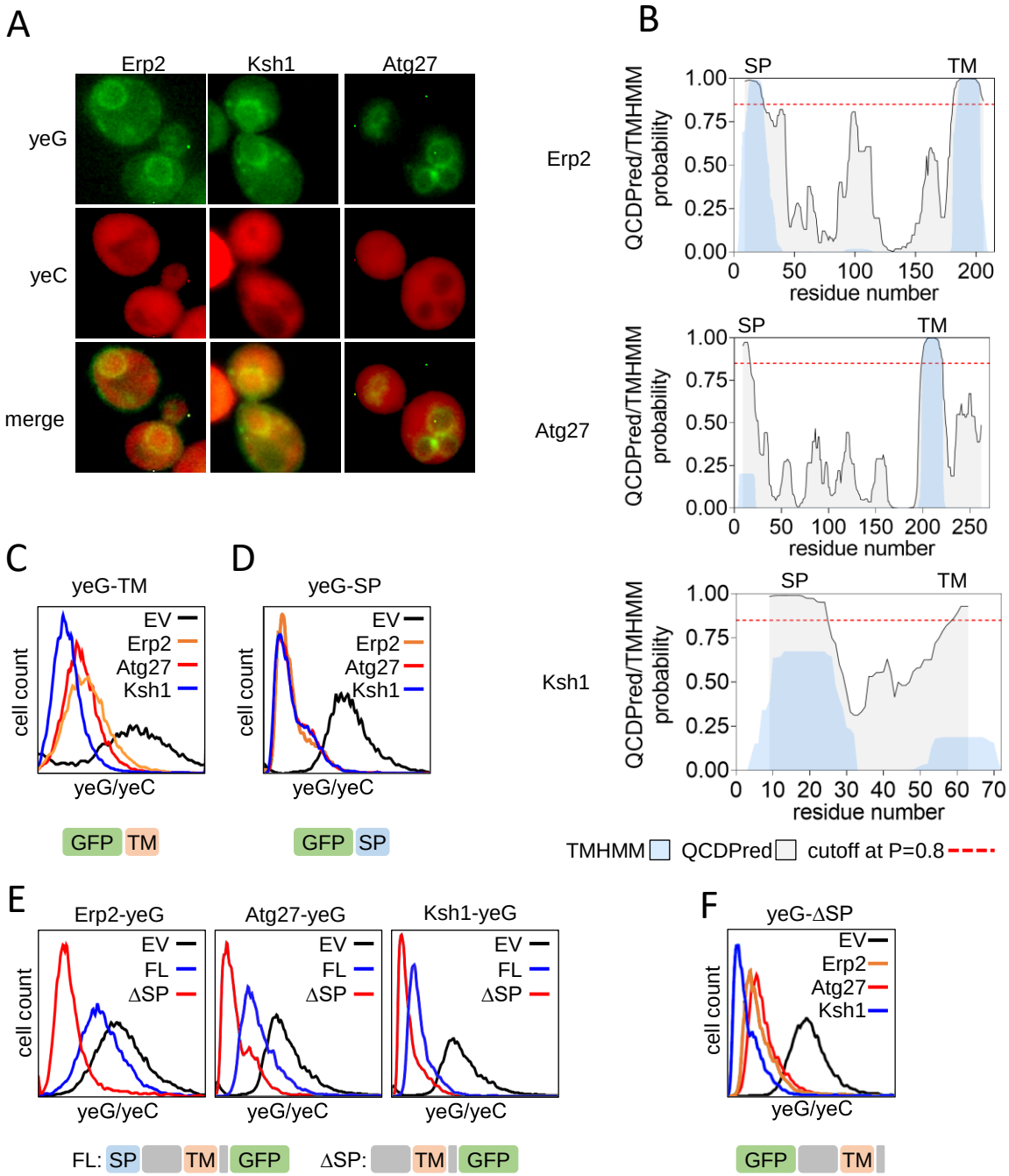
Pca1
QCDPred
TMHMM
QCDPred/TMHMM probability
1.00
0.75
0.50
0.25
0.00
0
300
600
900
1200
residue number
B
degrons
TMDs
>95.1%
C
QCDPred
TMHMM
overlay
D
(i) all
(ii) TMD
(iii) non TMD
frequency (%)
20
15
10
5
0
0.85-0.86
0.87-0.88
0.89-0.9
0.91-0.92
0.93-0.94
0.95-0.96
0.97-0.98
0.99-1
QCDPred probability ranges

Figure 4


A
Erp2
Ksh1
Atg27
yeG
yeC
merge
B
Erp2
Atg27
Ksh1
SP
TM
QCDPred/TMHMM probability
residue number
TMHMM
QCDPred
cutoff at P=0.8
C
yeG-TM
D
yeG-SP
EV
Erp2
Atg27
Ksh1
cell count
yeG/yeC
GFP
TM
SP
E
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Atg27-yeG
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FL
ΔSP
FL:
ΔSP:
F
yeG-ΔSP

Figure 5

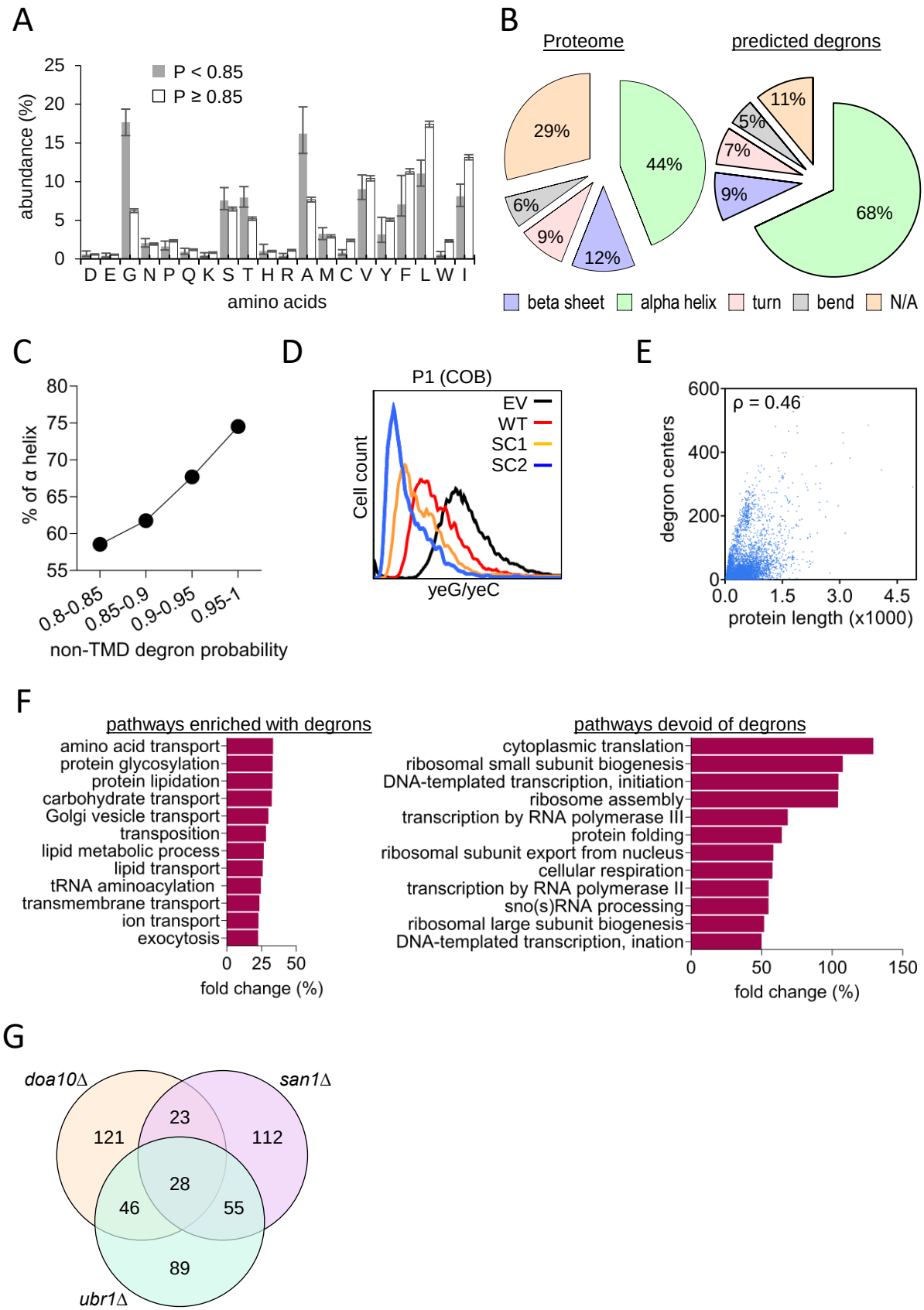
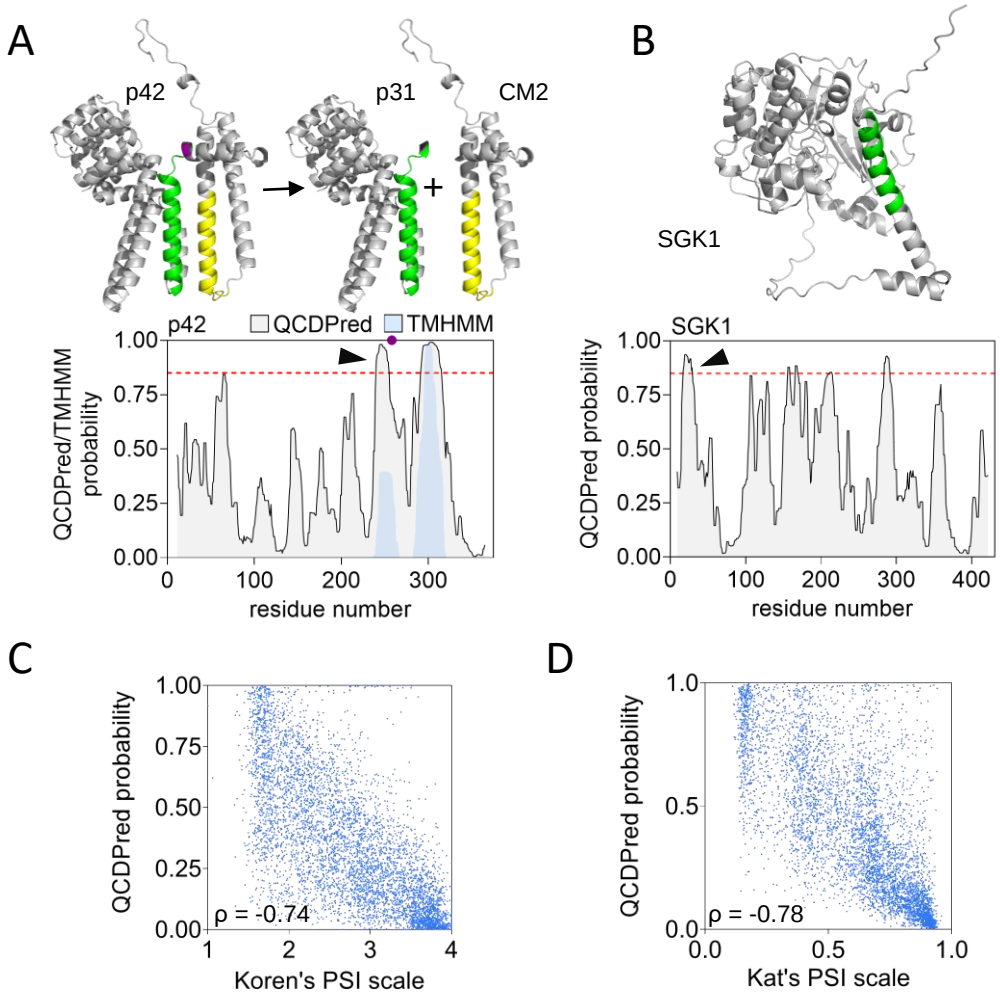
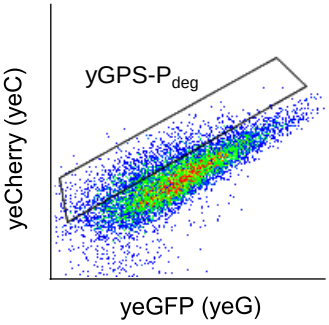

A
P < 0.85
P ≥ 0.85
abundance (%)
D E G N P Q K S T H R A M C V Y F L W I
amino acids
B
Proteome
predicted degrons
29%
44%
6%
9%
12%
11%
5%
7%
9%
68%
beta sheet
alpha helix
turn
bend
N/A
C
% of α helix
0.8-0.85
0.85-0.9
0.9-0.95
0.95-1
non-TMD degron probability
D
P1 (COB)
EV
WT
SC1
SC2
Cell count
yeG/yeC
E
ρ = 0.46
degron centers
protein length (x1000)
F
pathways enriched with degrons
amino acid transport
protein glycosylation
protein lipidation
carbohydrate transport
Golgi vesicle transport
transposition
lipid metabolic process
lipid transport
tRNA aminoacylation
transmembrane transport
ion transport
exocytosis
fold change (%)
pathways devoid of degrons
cytoplasmic translation
ribosomal small subunit biogenesis
DNA-templated transcription, initiation
ribosome assembly
transcription by RNA polymerase III
protein folding
ribosomal subunit export from nucleus
cellular respiration
transcription by RNA polymerase II
sno(s)RNA processing
ribosomal large subunit biogenesis
DNA-templated transcription, ination
fold change (%)
G
doa10Δ
san1Δ
ubr1Δ
121
23
112
28
46
55
89

Figure 6


A
p42
p31
CM2
+
p42
QCDPred
TMHMM
QCDPred/TMHMM probability
1.00
0.75
0.50
0.25
0.00
0
100
200
300
residue number
B
SGK1
SGK1
QCDPred probability
1.00
0.75
0.50
0.25
0.00
0
100
200
300
400
residue number
C
QCDPred probability
1.00
0.75
0.50
0.25
0.00
ρ = -0.74
1
2
3
4
Koren's PSI scale
D
QCDPred probability
1.0
0.5
0.0
ρ = -0.78
0.0
0.5
1.0
Kat's PSI scale

Supplemental Figure 1, Isolation of top 10% degrons

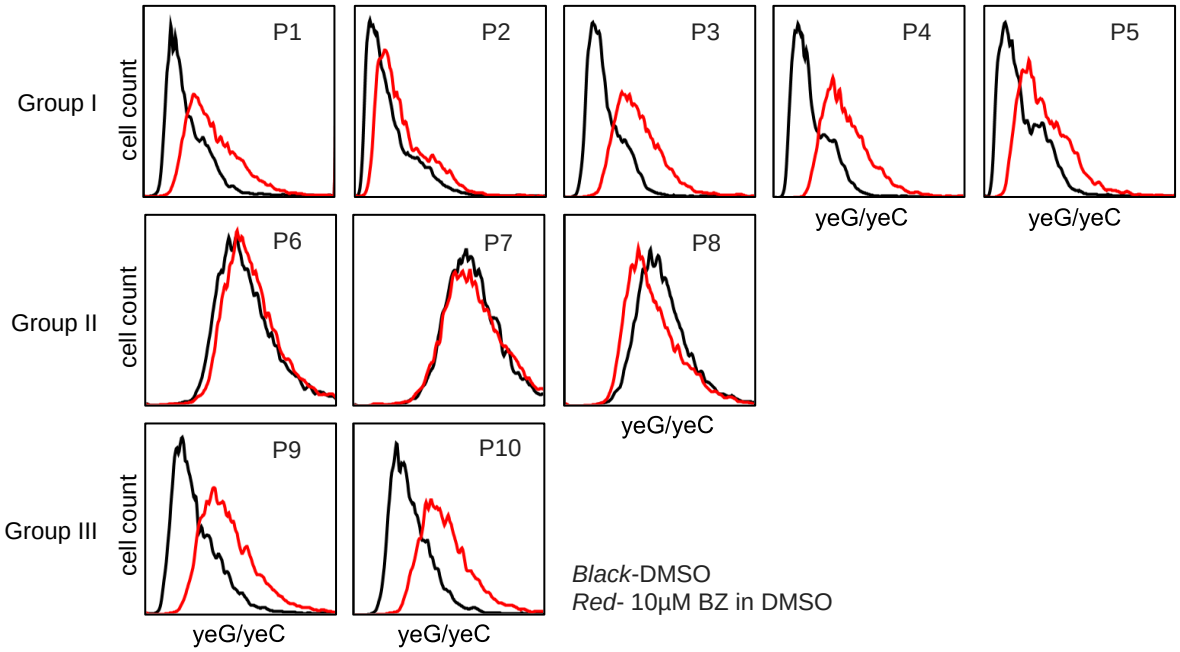


Supplemental Figure 1, related to Figure 1. Isolation of top 10% degrons. Yeast cells expressing yGPS-P_{lib} Were grown to mid-log-phase, followed by FACS of top 10% degron population. One million cells comprising the degron library (yGPS-P_{deg}) were collected, then grown O/N and frozen in liquid nitrogen or taken for further analysis. Illustrated is gating of top 10% degron population out of a total of 10,000 cells.

Supplemental Figure 2

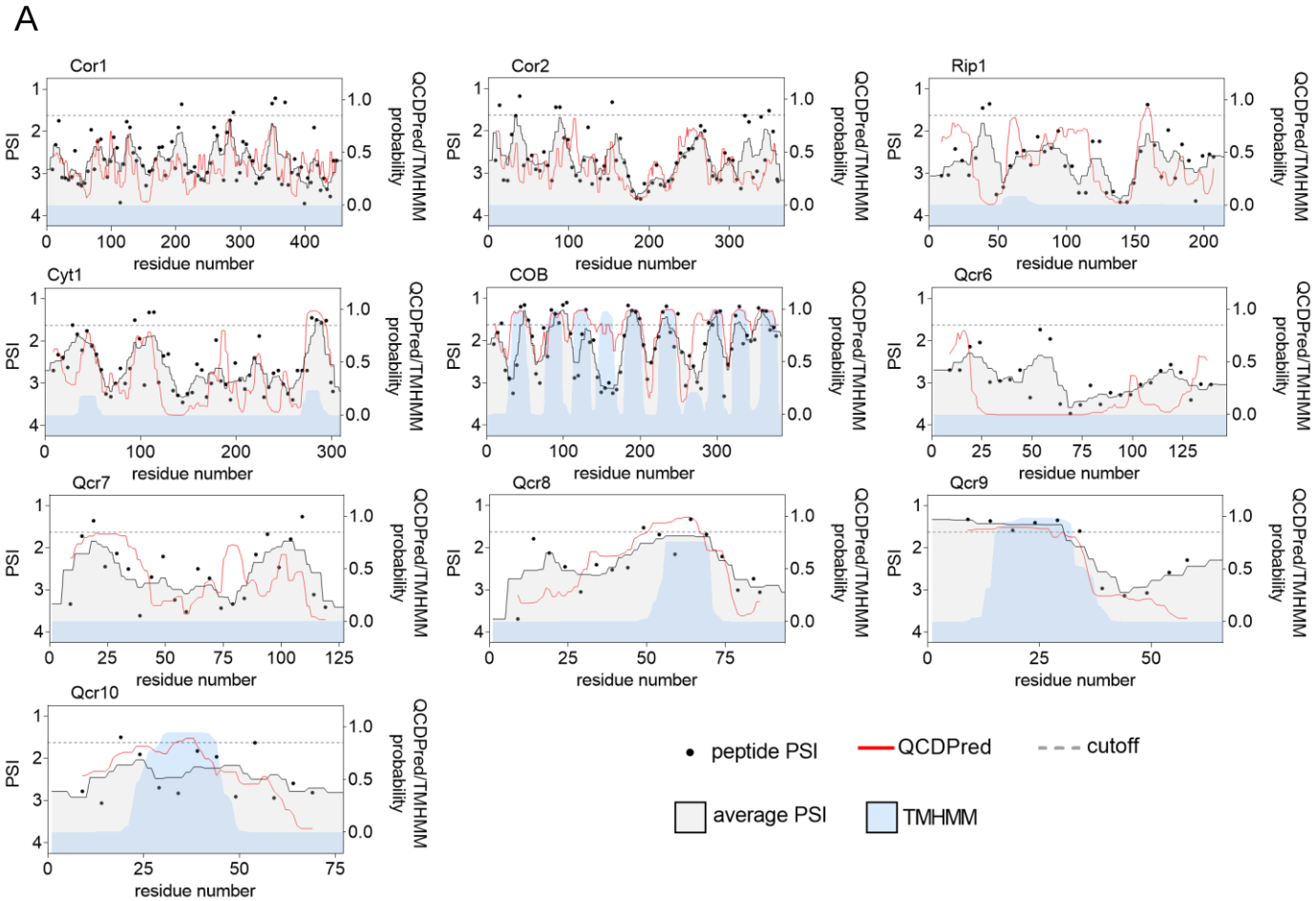
Supplemental Figure 2. **related to Figure 2**. Maps of PSI values emerged from experimental data (black dots), average PSI values for each amino acid (colored in black) and calculated QCDPred probabilities (colored in red) were plotted for each of the proteins in the screen that reach 100% coverage (N=306).

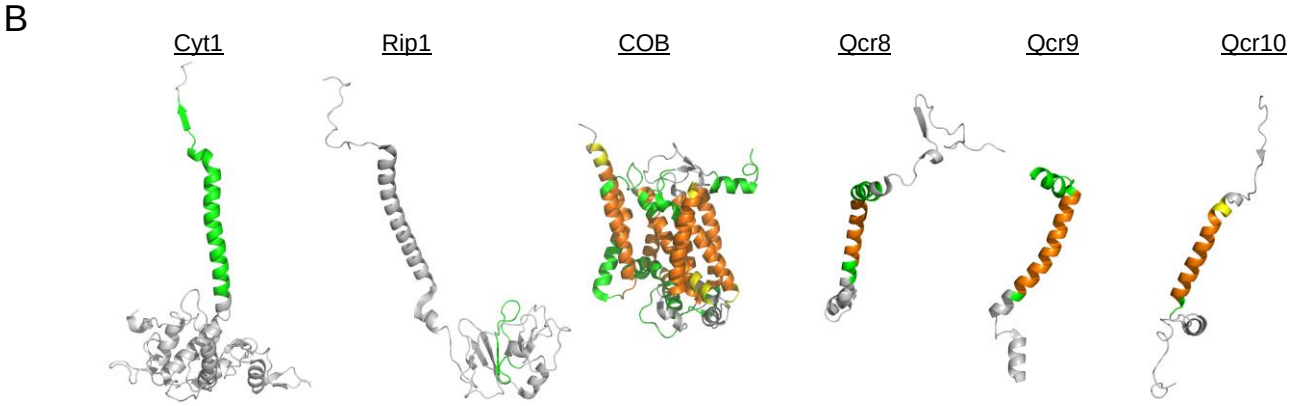
Supplemental Figure 3



Supplemental Figure 3, related to figure 2. Group-I and group-III peptides are substrates of the proteasome. Cells expressing the related fusion peptides were incubated with DMSO (vehicle) or with 10µM Bortezomib (BZ) for four hours. The increased yeG/yeC in group-I and group-III peptides indicate proteasome-dependent degradation. No significant effect of BZ on Group-II peptides was observed.

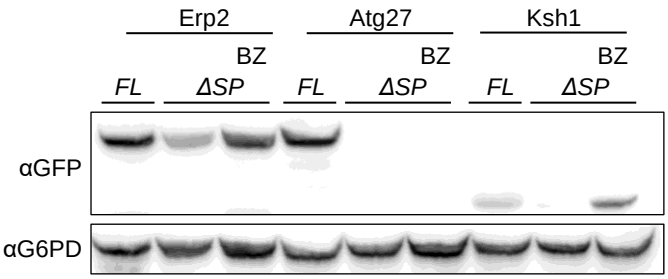
Supplemental Figure 4. Degron prediction in the b-c1 complex





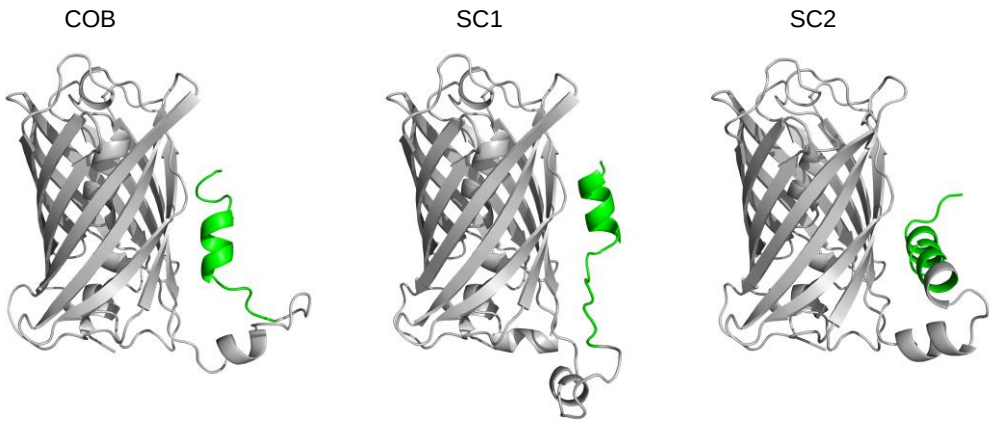
Supplemental Figure 4, related to figure 3. Degrons in the b-c1 complex. (A) PSI values emerged from experimental data (black dots), average PSI values for each amino acid (colored in black), QCDPred probabilities (colored in red), and TMHMM prediction (colored in light blue) were plotted for each subunit of the b-c1 complex. A degron cutoff probability is marked at P = 0.85 by a dashed grey line. (B) Assignment of QCDPred projected Degrons (green) and TMHMM predicted TM domains (yellow) to TM proteins in the b-c1 3D structure (PDB #6t0b). Overlying regions are colored in orange.

Supplemental Figure 5.



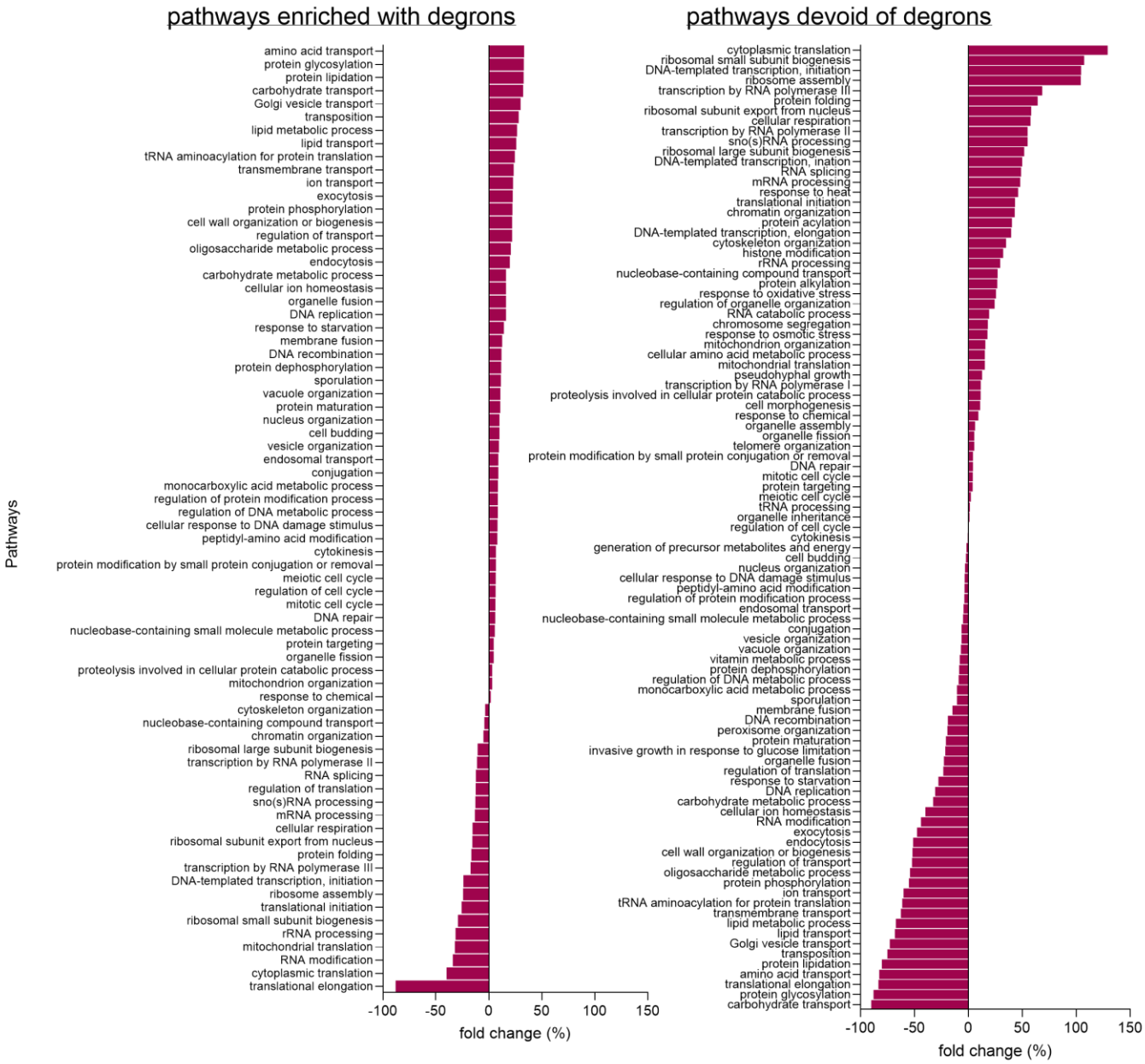
Supplemental Figure 5, related to Figure 4. Immunoblot analysis of Erp2, Atg27 and Ksh1, with or without their SPs. Where indicated, 10 µM of Bortezomib (BZ) were added to cells for 4 hrs prior to cell harvesting. *FL*- full length proteins. Δ*SP*- proteins devoid of the SP. G6PD: Glucose-6-phosphate dehydrogenase

Supplemental Figure 6



Supplemental Figure 6, related to Figure 5. Structure prediction of GFP-fused peptides using trRosseta. Estimated Template Modeling scores: COB-0.856, SC1-0.865, SC2-0.828

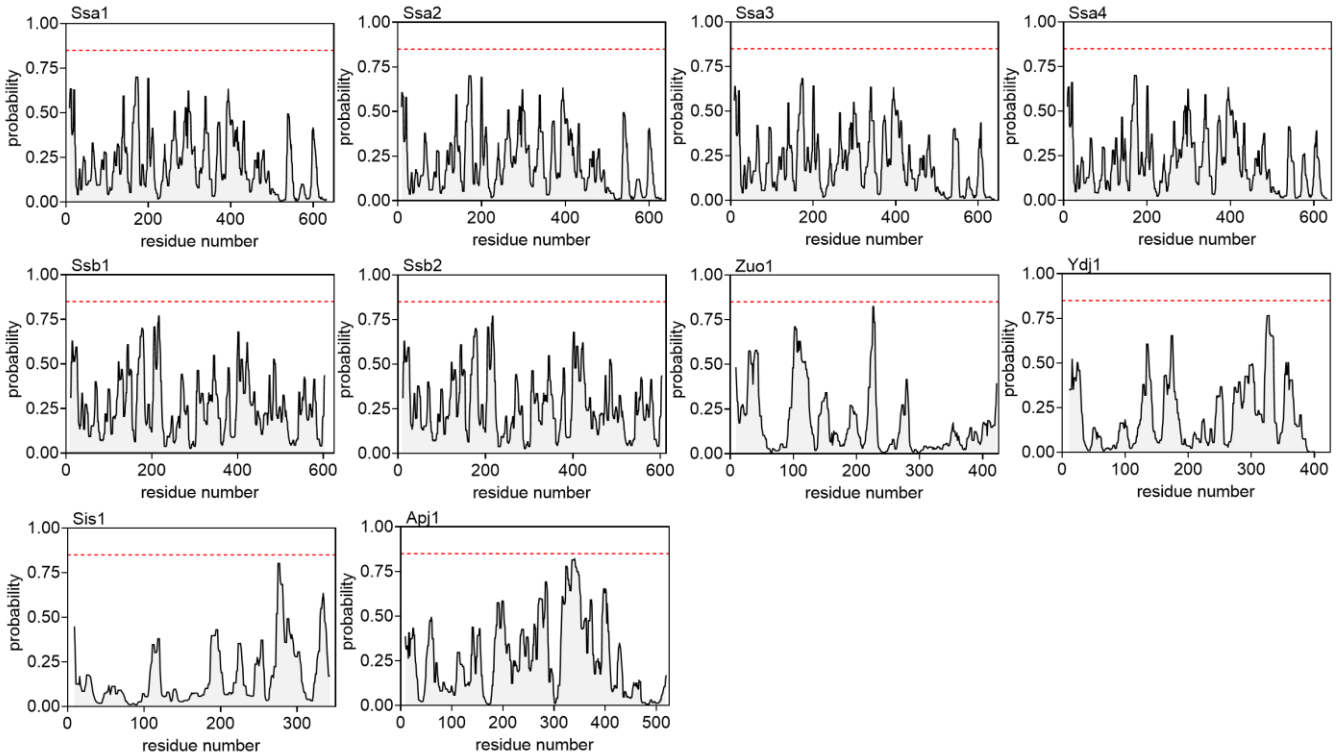
Supplemental Figure 7



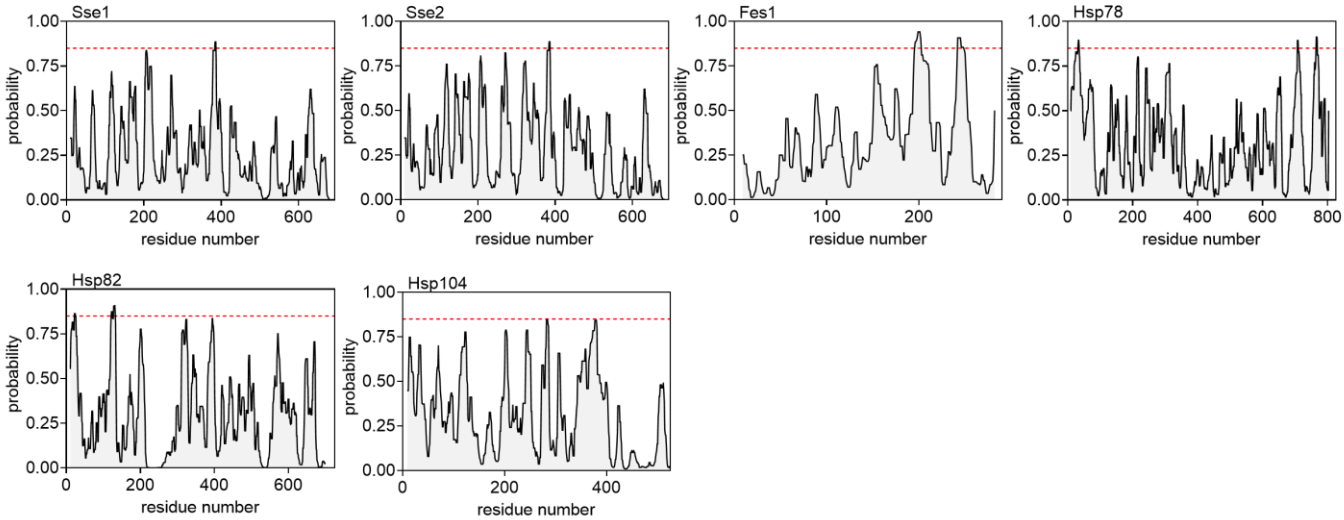
Supplemental Figure 7, related to Figure 5. Large-scale GO annotations of all significant processes (p-value < 0.05) that are either enriched or devoid of predicted degrons at $P \geq 0.85$.

Supplemental Figure 8

Cytosolic/nuclear Hsp40/70 chaperones



Cytosolic/nuclear Hsp90/100/110 chaperones



Supplemental Figure 8, related to figure 5. QCDPred probability maps of key cytosolic/nuclear yeast chaperones. A degron cutoff probability is marked at P = 0.85 by a dashed red line.