The N-end Rule Pathway and Ubr1 enforces the compartmentalization of mitochodrial and secretory proteins through the recognition of P2-encoded cellular location signals

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ABSTRACT

Cells have evolved intricate systems of protein quality control (PQC) that serve to monitor the cell for misfolded proteins and target them for degradation, ameloriating the effects of the majority of folding failures. In recent years, Ubr1, an E3 ligase conserved from yeast to humans, has been demonstrated to be involved in the degradation of several misfolded substrates within the cytosol through the N-end rule pathway, but the significance of this activity was not completely understood. Through a systematic examination of P2-residue variants of model misfolded substrates, followed by global and targeted analyses of yeast proteins, we have determined that Ubr1 is specifically programmed to preferentially target mistranslocated secretory and mitochondrial proteins in the cytosol through the recognition P2-encoded cellular location signals. Recognition is partially dependent on whether a protein is misfolded, and hence more likely to disrupt homeostasis in the cytosol. This newfound N-degron-dependent mechanism for maintaining cytosolic proteomic integrity may shed light on how Ubr1 and the N-end rule pathway are involved in the progression of neurodegenerative diseases and other maladies linked to the accumulation of misfolded proteins. A corollary to this discovery is that the N-end rule pathway in eukaryotic cells enforces the compartmentalization of proteins by degrading those that fail to reach their intended sub-cellular locations. Thus, the N-end rule pathway is likely to have also played a critical role in the evolution of endosymbiotic relationships which paved the way for advanced eukaryotic cellular life.

Protein quality control (PQC) is an essential protein quality surveillance and degradation system through which cells ensure the integrity of the proteome and maintain cellular homeostasis (Kim et al., 2013). Uncontrolled aggregation of misfolded proteins leads to the formation of insoluble protein deposits that are detrimental to the cell and are widely associated with numerous human diseases such as Alzheimer's, Parkinson's, and Huntington's disease (Currais et al., 2017; Dubnikov et al., 2016; Pearce and Kopito, 2017). Cytosolic quality control (CytoQC) pathways, a subset of PQC, specifically mediates the clearance of cytosolically-localized aberrant proteins. Previous studies have linked the Met-Φ branch of the N-end rule pathway to CytoQC, while the same has been suggested for the Ac/N-end branch (Kim et al., 2014; Kim et al., 2013; Shemorry et al., 2013; Eisele and Wolf 2008). However, the extent and role of the N-end rule pathway's participation in the context of CytoQC has not been fully understood. We and others have shown that detection of misfolded proteins are facilitated by the recognition of a specific set of Ubr1-QC-compatible P2-encoded amino acid residues, a subset of which produce Met-Φ N-degrons (Kim et al., 2014; Tran, 2013). Here, we expand on our previous findings, showing through a global bioinformatic analysis of the *S. cerevisiae* proteome that Ubr1, an E3 ligase of the N-end rule pathway, is designed to preferentially target to degradation secretory and mitochondrial proteins that are mistranslocated and thus become mislocalized to the cytosol. Furthermore, our analysis of a representative set of signal-sequence bearing proteins revealed that ~93% of soluble ER proteins are encoded with Ubr1-QC-compatible P2-residues, versus only ~26% of natively localized cytosolic proteins, indicating that P2-residues serve as general signals of cellular location. Our findings also strongly suggest that the P2-encoding of cellular location helps to enforce the fidelity of protein compartmentalization in eukaryotic cells.

In yeast, San1p and Ubr1p are the primary E3s in CytoQC (Prasad et al., 2010; Heck et al., 2010; Buchberger et al., 2010; Fredrickson and Gardner, 2012). Ubr2p, Doa10p, and Hul5p play less prominent roles (Ast et al., 2014; Buchberger et al., 2010; Nillegoda et al., 2010; Metzger et al., 2008; Fang et al., 2011). San1p targets substrates with exposed hydrophobicity consisting of contiguous sequences of at least five or more hydrophobic residues (Fredrickson et al., 2011, 2013; Fredrickson and Gardner 2012). It was demonstrated that a Ubr1 CytoQC substrate was recognized through an N-end rule pathway N-degron (Kim et al., 2014). Two other model misfolded substrates, Ste6*C and Δ2GFP, are degraded through a combination of the two pathways. Efficient degradation of each substrate requires both San1p and Ubr1p E3 ligases with differing dependencies: the degradation of Ste6*C is biased towards the Ubr1 pathway, whereas the degradation of Δ2GFP is biased towards the San1 pathway (**Figures 1A-B;** Tran, 2013; Prasad et al., 2012; Prasad et al., 2010). Experiments we performed previously revealed that a leading methionine followed by specific amino acids at the P2 position could enable efficient degradation of misfolded substrates by Ubr1, as demonstrated by mutating the P2 residues of the misfolded substrates (**Figures 1C-1E, 2A, S1A, S1B**; Tran, 2013). These findings were later

corroborated in work published by the Varshavsky Lab (Cal Tech) in 2014, which formally defined Met-Φ sequences (Φ: bulky hydrophobic residues) as an official class of N-end rule pathway N-degrons (Kim et al, 2014). In addition, our work also characterized additionals set of N-degrons that existed: 1) a leading Met residue followed by His, Lys, Arg, or Gln and 2) N-terminal Val and Gly residues (after initiating-Met cleavage). Those original findings are presented in the figures here, excerpted from the original work.

We observed that nine amino acids at the P2 position resulted in significant inhibition of Ubr1-mediated degradation of Ste6*C in Δ*san1*+UBR1 cells: Ala, Asn, Ser, Cys, Glu, Pro, Asp, Thr, and Met, which we refer to as Ubr1-QC-*incompatible* P2 amino acids. His, Trp, Phe, Tyr, Ile, Val, Gln, Gly, Lys, Leu, and Arg, allowed rapid degradation by Ubr1, which we refer to as Ubr1-QC-*compatible* P2 amino acids **(Figure 2A, S2A-S2C)**. The same effect was not seen in +SAN1Δ*ubr1* cells, for which no statistically significant change to turnover rate relative to the original Ste6*C substrate was observed (**Figure 2A, S2D-S2F**). The Ubr1-dependent degradation of the 9 most rapidly degraded Ste6*C P2 mutants was confirmed in Δ*san1*Δ*ubr1* cells (**Figure S3**). Ste6*C P2 mutants inhibited for Ubr1 degradation was not a result of Ubr1 blockage by N-terminal acetylation since Ste6*C is encoded with a P3 Pro, which is known to prevent N-terminal acetylation (Polevoda and Sherman, 2003). Leading methionine retention is not a prerequisite for compatibility with the Ubr1-QC degradation pathway, as Ste6*C-I2V was demonstrated to be degraded efficiently by Ubr1 and is predicted to undergo methionine cleavage, also confirmed via sequencing (**Figure S1C**).

Interestingly, P2 His, Lys, Gln, and Arg, all of which cause retention of initiating methionines based on the rules of methionine excision (Sherman et al., 1985), are not predicted to produce N-termini with known N-degrons of the N-end rule pathway (Kim et al, 2014). Likewise, P2 Gly and Val residues, which lead to the cleavage of leading methionines and become exposed have also not been identified as N-degrons. Thus, our experiments demonstrate that P2 His, Lys, Gln, Arg, Gly, and Val residues generate novel N-terminal N-degrons recognized by Ubr1. These differences suggest that there are context and substrate specific influences on the N-end rule pathway's activity in CytoQC. Thus far, the expanded set of N-degrons discovered using Ste6*C applies to misfolded proteins in the cytosol, but may possibly extend to other targets and physiological states not explored here.

The majority of secretory pathway and mitochondrially-localized proteins encode Ubr1-QC-compatible P2 residues

To determine if there is a bias within specific cellular compartments for proteins to possess P2 residues that are Ubr1-QC-compatible, we utilized data from a genome-wide GFP-fusion based localization study of protein localization spanning 4156 proteins (**Table S2-S4**; Huh et al., 2003). P2 amino acid usage frequency was analyzed for proteins with exclusive nuclear, cytosolic,

secretory pathway, or mitochondrial localization (**Tables S5-S8; Refer to Materials and Methods**). Frequency distribution across all residues was significantly different for secretory (n=559, $p < 1 \times 10^{-6}$, $x^2 = 64.33$, $df = 19$) and mitochondrial proteins (n=458, $p < 1 \times 10^{-55}$, $x^2 = 317.85$, $df = 19$) when compared against cytosolic proteins (n=823) (**Figures 2B-D; Tables S9 and S10**). Pair-wise chi-square analysis of individual amino acid usage frequencies demonstrated that biases were seen for the mitochondrial and secretory proteins to be encoded with P2 residues that are Ubr1-QC-compatible, with 6 Ubr1-QC-compatible P2 amino acids (Trp, Phe, Ile, Lys, Leu, and Arg) having statistically significant higher percentage usage frequencies in at least one or both of the mitochondrial and secretory pathway protein sets when compared with cytosolic proteins. An equally important finding is that we observed a significant usage bias *against* 7 amino acids that do *not* enable efficient Ubr1 degradation (Ubr1-QC-*incompatible* P2 residues Ala, Ser, Cys, Glu, Pro, Asp, Thr) in either one or both mitochondrial and secretory pathway protein sets when compared to cytosolic proteins. In contrast, frequency distribution between nuclear and cytosolic proteins had a much less significant difference (nuclear, n=639; cytosolic n = 823; $p > 0.001$, $x^2 = 36.33$, $df = 19$), and only a few significant differences in relative frequencies of individual amino acids. No general bias for or against Ubr1-QC P2 compatibility was observed between the two groups (**Figure 2E; Table S10).**

We determined the total relative abundance of proteins within each localization category based on abundance level data gathered from the global yeast protein GFP-fusion study conducted by Huh and colleagues (refer to Materials and Methods). The majority of nuclear and cytosolically localized protein is encoded with Ubr1-QC-*incompatible* P2 amino acids (84.7% and 70.9%, respectively), while the majority of secretory and mitochondrial protein is associated with Ubr1-QC-*compatible* P2 amino acids (59.7% and 82.3%, respectively) **(Figure 2F; Table S17-S27)**.

The much higher prevalence of Ubr1-QC-compatible proteins in the latter two categories indicated that Ubr1p may be optimized for the degradation of secretory and mitochondrial protein. That they do not natively reside in the cytosol suggested a model in which proteins in these pathways are subject to Ubr1-mediated CytoQC when they fail to translocate. Compartment-specific chaperones and enzymes are important for the native folding processes of secretory and mitochondrial proteins (Hartl et al., 2011; Haynes and Ron, 2010; Stevens and Argon, 1999). Spontaneous folding by mitochondrial proteins in the cytosol has been shown to occur, thereby preventing import (Strobel, 2002). Various cytosolic chaperones are designed to maintain pre-translocated proteins in partially-folded, import-competent states, while the folding efficiency of signal-sequence carrying precursor proteins has been to shown to be significantly lower than for that of their mature, signal-sequence cleaved counterparts (Neupert,1997; Laminet and Pluckthun, 1989). Thus, a translocation failure of mitochondrial and secretory proteins should theoretically generate conditions optimal for recognition and degradation by Ubr1p: availability of an uncleaved N-terminal sequence containing an N-degron accompanied by impaired or inhibited folding.

Mis-translocated secretory and mitochondrial proteins are degraded by the N-end rule pathway

To test the above hypothesis, we examined ATP2Δ1,2,3, a translocation-defective mutant of Atp2p that is import-deficient and degraded in the cytosol (Bedwell et al., 1987). ATP2Δ1,2,3 carries a Ubr1-QC-compatible P2 Val residue. Degradation was dependent on a combination of San1 and Ubr1 **(Figure 3A).** We substituted the P2 Val with Ser and observed an inhibition of Ubr1-mediated degradation **(Figure 3B)**. The P3 Leu residue was mutated to Pro to inhibit acetylation (Polevoda and Sherman, 2003). Both forms of the substrate were stabilized to similar levels in *Δsan1Δubr1* cells. Degradation was also dependent on Sse1, Ydj1, and Ssa1 and Ssa2 chaperones **(Figure S4)**, hallmarks of UPS-dependent CytoQC. Degradation of wild-type Atp2p in a temperature sensitive mitochondrial-import mutant, *tom40-2,* was dependent on Ubr1 (Krimmer et al., 2001) **(Figure S5).** These results confirm that translocation-deficient Atp2p is an endogenous substrate of Ubr1-mediated CytoQC.

To determine if translocation-defective secretory pathway proteins are also subject to Ubr1 CytoQC, we generated an import-impaired mutant of vacuolar carboxypeptidase Y (CPY), CPY-12iE, in which a Glu residue is inserted at position 12 to disrupt the hydrophobic core of the native CPY signal sequence. Since any mature, vacuole-processed CPY-12iE would prevent accurate analysis of its pre-form levels, we utilized the fact that C-terminal processing of CPY-HA results in HA tag removal upon maturation **(Figure S6).** Degradation of pre-CPY-12iE was significantly reduced by the absence of Ubr1p (**Figure 3C**, *Δsan1*+UBR1 vs *Δsan1Δubr1).* The degradation of endogenous pre-forms of GAS1 and CPY proteins in ER-import deficient strain *sec63-1* was dependent on Ubr1, demonstrating that Ubr1 is required for the efficient degradation of a subset of mis-translocated endogenous secretory proteins (personal communication with Ng Lab; unpublished data). Both CPY and GAS1 are encoded with Ubr1-QC-compatible P2 residues, Lys and Leu, respectively.

To assess the significance of a substrate's misfoldedness to Ubr1-recognition of the substrate via a Ubr1-QC-compatible P2 residue, we examined wild-type GFP, which is expected to escape CytoQC-based degradation. The P2 Ser of GFP was substituted with Ile to generate GFP-S2I. GFP-S2I was significantly less susceptible to Ubr1 compared to Δ2GFP-S2I, which was degraded efficiently by Ubr1 **(Figure 3F).** Trypsin digestion confirmed the relative structural stability of GFP-S2I when compared to Δ2GFP-S2I **(Figure 3G).** These results indicate that degradation of a substrate via the N-end rule pathway is significantly enhanced when the substrate is also misfolded. This may be a result of the N-terminus of a folding-compromised substrate being aberrantly exposed, either spatially or temporally, making binding by Ubr1p binding more kinetically favorable. This is similar to the model of protein quality control proposed for proteins possessing Ac/N-degrons. (Shemorry et al., 2013; Hwang et al., 2010).

Alternatively, it may be due to an increased availability of a misfolded substrate's polyubiquitination-competent lysine residues, as one of the requirements of Ubr1-dependent degradation is access to a lysine in an unstructured region of the substrate (Varshavsky, 2011).

P2 residues act as cellular location signals that mediate CytoQC via the N-end rule pathway

Studies have shown that mislocalized membrane proteins in the cytosol are targeted for degradation through exposed hydrophobic patches in both yeast and mammalian a cells (Hessa et al., 2011; Kawahara et al., 2013; Rodrigo-Brenni and Hegde, 2012; Ast et al., 2014; Suzuki & Kawahara, 2016). If mistranslocated proteins *without* Ubr1-QC-compatible P2 residues are primarily membrane proteins, most would be degradable through these pathways and rendered benign. To assess whether Ubr1 N-end rule based CytoQC, along with quality control pathways targeting hydrophobicity, could together provide an effective degradation system for clearing the cytosol of mis-localized proteins, we analyzed a set of 277 ORFs encoding signal sequence-containing proteins used in a previous study to determine the frequency of amino acids encoded at the P2 position in secretory proteins (Forte et al., 2011). This set was filtered for duplicates and other inconsistencies, resulting in 273 proteins which were then categorized as soluble or membrane proteins (refer to Materials and Methods). Strikingly, 87.5% (49/56) of ER proteins encoded with Ubr1-QC-*incompatible* residues are membrane proteins, strongly differing from the full set of signal-sequence bearing proteins, for which only 64.1% (173/270) are membrane proteins ($p < 0.001$, $x^2 = 11.7$) (**Figure 4B; Tables S12-S16)**. An even larger difference was observed when compared to the relative frequency of membrane proteins in the set of Ubr1-QC-compatible P2-residue-encoded proteins (57.9% (124/214); $p < 5 \times 10^{-5}$, $x^2 = 16.8$). These results indicate that the vast majority of ER proteins which are not compatible with Ubr1 are also, at a higher relative frequency, membrane proteins, which is expected to allow their degradation through pathways that target exposed hydrophobicity, such as San1-mediated CytoQC.

Interestingly, statistically significant differences were seen in the relative frequency of proteins encoding Ubr1-QC-compatible P2 residues when comparing the set of soluble proteins with membrane proteins (Chi-square test: $p < 5 \times 10^{-5}$, $x^2 = 16.8$), as well as with the full set of signal-sequence bearing proteins ($p < 0.005$, $x^2 = 9.7$): 92.8% (90/97) of the soluble protein set possess Ubr1-QC-compatible P2 residues, versus 78.8% (215/273) of the full set of signal-sequence bearing proteins, and only 71.7% (124/173) of the membrane proteins. (**Figure 4A; Table S12-S16**). Thus, while the majority of signal-sequence bearing proteins are susceptible to Ubr1, soluble secretory proteins are particularly suited for degradation through this pathway. In contrast, only ~26.6% of cytosolic proteins possess Ubr1-QC-compatible P2 residues based on an analysis of a representative set of 251 randomly selected cytosolic proteins (**Figure 4A**; **Table S11**; Forte et al., 2011). This minority of cytosolic proteins with Ubr1-QC-compatible P2 residues are expected to fold efficiently in their native environment, thereby escaping targeting

by Ubr1 CytoQC, as demonstrated in pulse-chase experiments involving folded vs misfolded GFP **(Figure 3F).**

The findings presented here broaden our understanding of how eukaryotic cells ensure the the cytosol is kept in a healthy state mostly clear of foreign actors that could disrupt normal cellular processes such as signaling, protein synthesis, and trafficking. Failures in SRP-mediated ER targeting causes the mistargeting of secretory proteins to the mitochondria, resulting in mitochondrial dysfunction (Costa et al., 2018), while depletion of nascent polypeptide-associated complex (NAC) results in the incorrect import of mitochondrial proteins into the ER lumen (Gamerdinger et al., 2015). Such aberrant cross-organelle mis-targeting would also be mitigated by the Ubr1 P2-dependent CytoQC pathway, capturing such proteins in the cytosol before mis-targeting is able to occur. We propose that there is an intrinsic pressure for secretory and mitochondrial proteins to be encoded with Ubr1-QC-compatible P2 residues so they can be efficiently degraded in the event of mislocalization as a result of translocation failure. Soluble ER proteins in particular are preferentially targeted by Ubr1. That they would not be recognized by hydrophobicity-based degradation pathways due to their lack of transmembrane domains, and possession of only moderately hydrophobic signal sequences, necessitates Ubr1-QC-compatibility to be efficiently cleared from the cytosol. Together, our findings also suggest that P2 residues are utilized as *de facto* cellular location signals which help to ensure the the fidelity of protein localization. This system of P2-encoded location signaling facilitates N-end rule pathway-mediated degradation of the majority of soluble mis-translocated secretory and mitochondrial proteins in the cytosol. Confoundingly, the localization of Ubr1 is predominantly in the nucleus, where some of its misfolded substrates are imported and ubiquitinated by it (Prasad et al. 2018). A smaller fraction of Ubr1 also operates on substrates in the cytosol itself (Stolz et al., 2013; Heck et al., 2010; Eisele and Wolf, 2008). Whether the majority of mislocalized P2-residue-dependent Ubr1 substrates are engaged by the cytosolic form of Ubr1, or are first trafficked to the nucleus and subsequently recognized by the the nuclear form of Ubr1, remains to be determined.

Herpes simplex virus 1-encoded microRNA has been shown promote the accumulation of β-amyloid through the inhibition of Ubr1 activity, while aggregation-prone fragments of neurodegeneration-associated TDP43, Tau, and α-synuclein, are substrates of the N-end rule pathway (Zheng et al., 2018; Bowser et al., 2013). An intriguing avenue of research would be to investigate if the P2-residue based degradation of mis-localized proteins by Ubr1 described here is mirrored in mammalian cells, and if so, whether disruption of the pathway is involved in the accumulation of such detrimental disease factors.

An additional surprising corollary to our discovery is that the N-end rule pathway, through the recognition of P2-encoded cellular location signals, plays a primary role in enforcing the compartmentalization of secretory and mitochondrial proteins that would otherwise adversely

affect critical cellular activities in the cytosol. It is widely accepted that mitochondria and plastids in eukaryotes evolved from endosymbiotic partnerships, whereby host prokaryotic cells engulfed ancient aerobic bacteria and gained the benefit of increased energy production, while the new cellular tenants were consequently shielded from the environment (Archibald, 2015). Over time, gene transfer from endosymbionts to the host cell genome occurred that conferred physiological fitness benefits, and evolved further into complex systems that utilized transit peptides and specialized import machinery to direct host-encoded proteins in the cytosol back to the endosymbiotic entity (Henze, 2001). However, how did early eukaryotes begin to deal with endosymbiont-derived proteins that could not make it to their final destination? Based on the findings presented here, we propose that the N-end rule pathway played a critical role in the evolution of advanced eukaryotic cells by ensuring that proteins intended for endosymbiotic organelles were effectively destroyed if they failed to transport correctly. In this way, the N-end rule may have been the key enabler for eukaryotic cells to fully exploit the benefits of possessing endosymbiotic organelles by compensating for the negative effects of mistargeted proteins.

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Author contributions. AT designed the studies, performed the experiments, and wrote the manuscript.

Competing interests. The author declares that there are no competing interests.

FIGURES

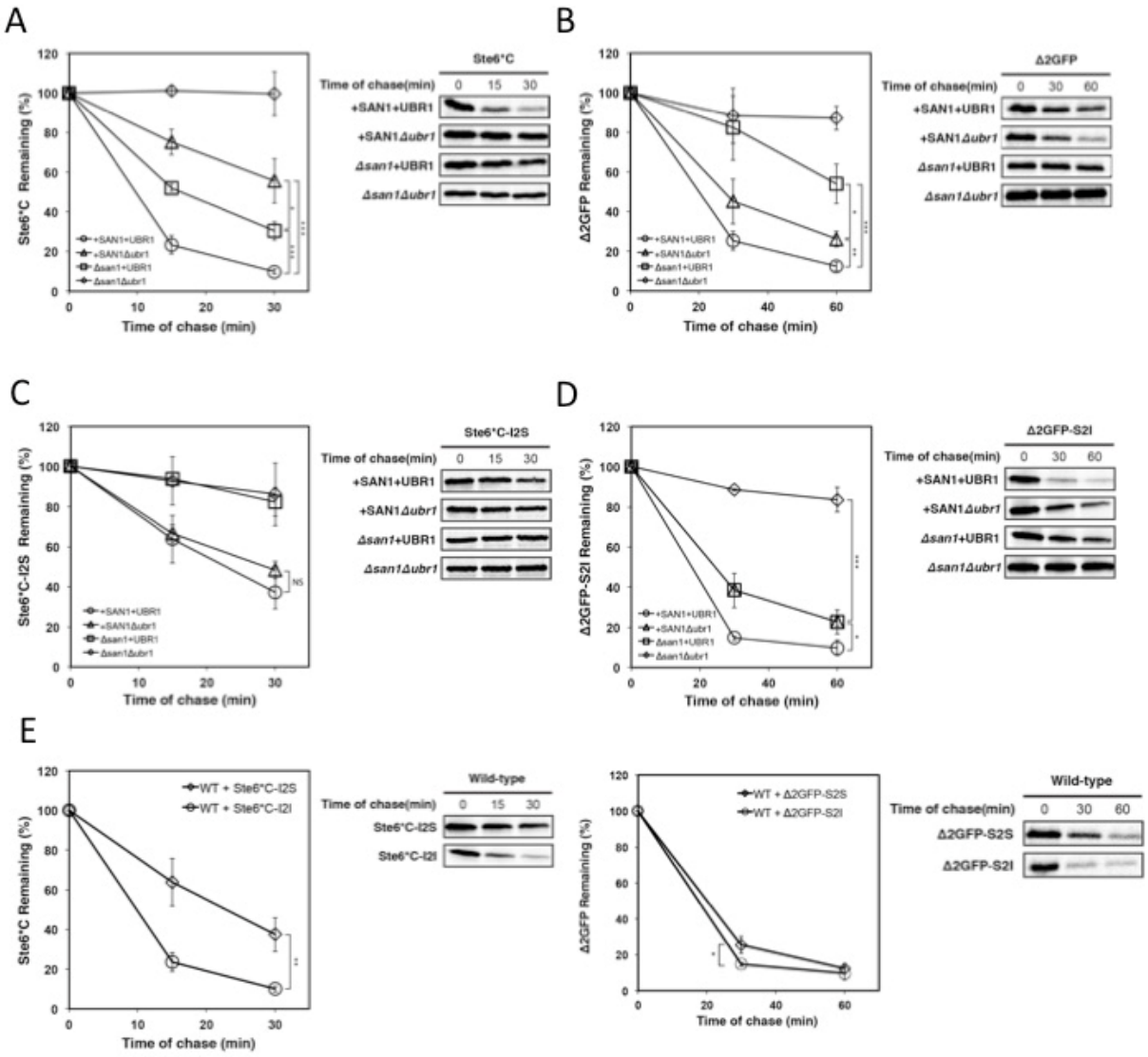

Figure 1. Efficient degradation of Ste6*C and Δ2GFP by the Ubr1 quality control pathway is dependent on P2 residue identity. (Figures adapted from Tran, 2013). (A) and (B) Turnover of Ste6*C and Δ2GFP in +SAN1+UBR1 (wild-type), Δsan1+UBR1, +SAN1Δubr1, and Δsan1Δubr1 cells by metabolic pulse-chase analysis. (C) and (D) Turnover of Ste6*C-I2S and Δ2GFP-S2I in +SAN1+UBR1(wild-type), Δsan1+UBR1, +SAN1Δubr1, and Δsan1Δubr1 cells was examined by metabolic pulse-chase analysis. (E) Comparison of turnover of P2-serine substrates (Ste6*C-I2S, Δ2GFP-S2S) with P2-isoleucine substrates (Ste6*C-I2I, Δ2GFP-S2I) in wild-type cells by metabolic pulse-chase analysis. (A-E) Metabolic pulse-chase: cells were grown to log phase and shifted to 30°C for 30 minutes followed by pulse-labeling with [35S]methionine/cysteine (5 min for Ste*6C and 10 min for Δ2GFP) and chased at the times indicated. Proteins were immunoprecipitated using anti-HA antibody and resolved by SDS- PAGE, then visualized by phosphoimager analysis. Error bars, mean +/- SD of at least three independent experiments. Student's t-test: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$; Not Significant (NS), $p > 0.5$.

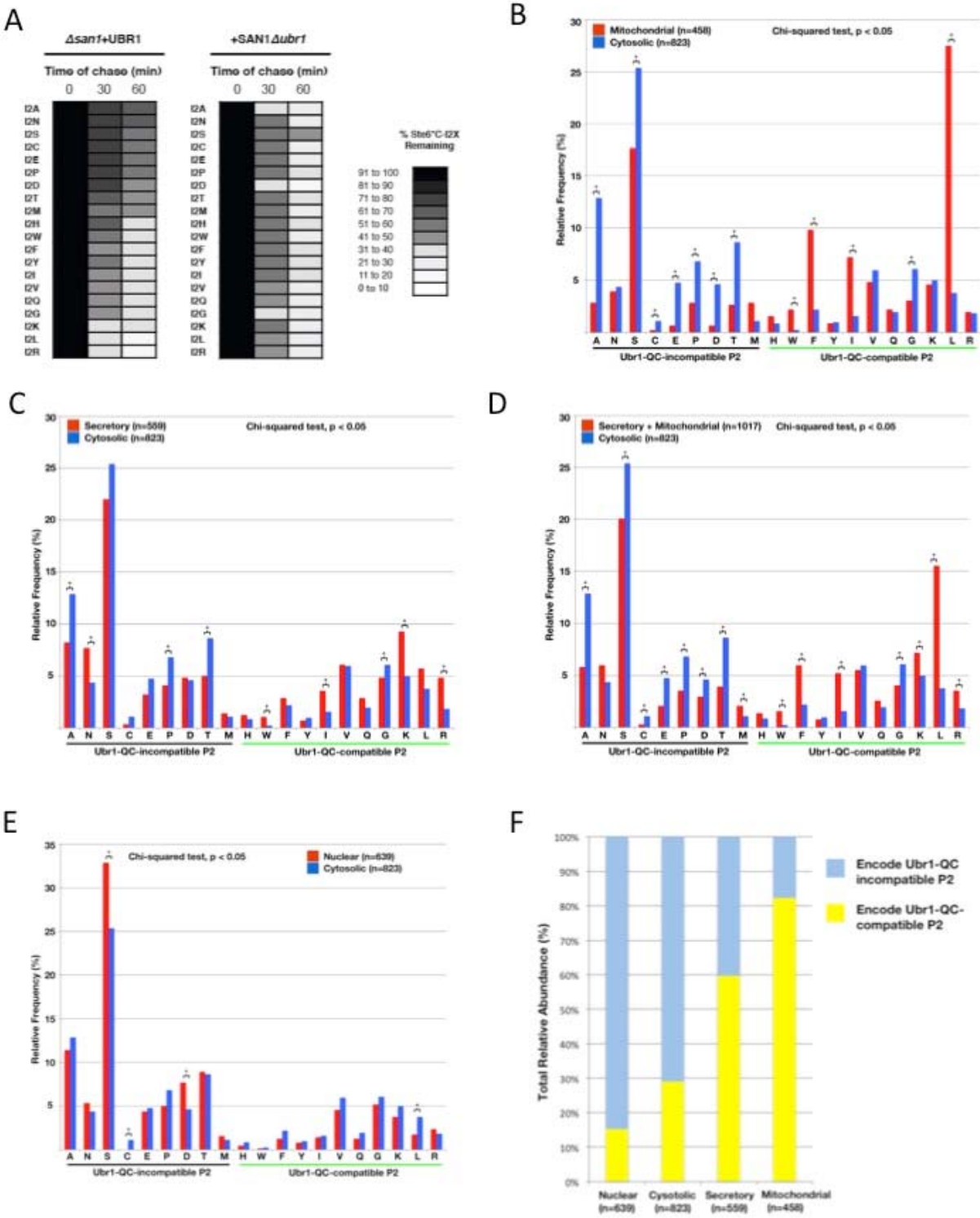
A
Δsan1+UBR1
+SAN1Δubr1
Time of chase (min)
0 30 60
I2A
I2N
I2S
I2C
I2E
I2P
I2D
I2T
I2M
I2H
I2W
I2F
I2Y
I2I
I2V
I2Q
I2G
I2K
I2L
I2R
% Ste6*C-I2X Remaining
91 to 100
81 to 90
71 to 80
61 to 70
51 to 60
41 to 50
31 to 40
21 to 30
11 to 20
0 to 10
B
Mitochondrial (n=458)
Cytosolic (n=823)
Chi-squared test, p < 0.05
Relative Frequency (%)
Ubr1-QC-incompatible P2
Ubr1-QC-compatible P2
C
Secretory (n=559)
Cytosolic (n=823)
Chi-squared test, p < 0.05
D
Secretory + Mitochondrial (n=1017)
Cytosolic (n=823)
Chi-squared test, p < 0.05
E
Nuclear (n=639)
Cytosolic (n=823)
Chi-squared test, p < 0.05
F
Total Relative Abundance (%)
Encode Ubr1-QC incompatible P2
Encode Ubr1-QC-compatible P2
Nuclear (n=639)
Cysotolic (n=823)
Secretory (n=559)
Mitochondrial (n=458)

Figure 2. The P2-residue identity of a CytoQC substrate specifically effects degradation efficiency of the Ubr1 quality control pathway. Proteins localized to secretory pathway components and mitochondria exhibit bias for encoding UBR1-QC-compatible P2 residues when compared to cytosolically-localized proteins. (A) Turnover rates of nineteen Ste6*C P2 amino acid residue mutants (Ste6*C-I2X, where X = A[alanine], N[asparagine], S[serine], C[cysteine], E[glutamic acid], P[proline], D[aspartic acid], T[threonine], M[methionine], H[histidine], W[tryptophan], F[phenylalanine], Y[tyrosine], V[valine], Q[glutamine], G[glycine], K[lysine], L[leucine], R[arginine]) were compared to the turnover rate of the original Ste6*C substrate (Ste6*C-I2I) in Δsan1+UBR1 and +SAN1Δubr1 cells by pulse chase analysis: cells were grown to log phase and shifted to 30°C for 30 minutes followed by pulse-labeling with [35S]methionine/cysteine for 5 minutes and chased at the times indicated. Proteins were immunoprecipitated using anti-HA antibody and resolved by SDS- PAGE, then visualized by phosphoimager analysis. Heat-map reflects results presented in Figures S2A, S2B, S2C, S2D, S2E, and S2F, and is based on the mean of at least three independent experiments. (Figure adapted from Tran, 2013) (B) Relative frequency of P2 amino acid usage of proteins localized to the secretory pathway components (n=559) vs cytosol (n=823). Frequency distribution differed significantly between the two sets ($p < 1 \times 10^{-6}$, $x^2 = 64.33$, $df = 19$; Table S9). Relative usage frequency of amino acids Ala (A), Asn (N), Pro (P), Thr (T), Trp (W), Ile (I), Lys (K), and Arg (R) differed significantly between the two sets (pair-wise Chi-square analysis; $p < 0.01$ for A, N, K, R; $p < 0.05$ for P, T, W, I). (C) Relative frequency of P2 amino acid usage in proteins localized to mitochondria (n=458) vs cytosol (n=823). Frequency distribution differed significantly between the two sets ($p < 1 \times 10^{-55}$, $x^2 = 317.85$, $df = 19$; Table S9). Relative usage frequency of amino acids Ala (A), Ser(S), Cys (C), Glu (E), Pro (P), Asp (D), Thr (T), Trp (W), Phe (F), Ile (I), Gly (G), and Leu (L) differed significantly between the two sets (pair-wise Chi-square analysis; $p < 0.0001$ for A, S, E, P, D, T, F, I, L; $p < 0.05$ for C, W, G) (D) Relative frequency of P2 amino acid usage in proteins localized to either mitochondria or secretory pathway (n=1017) vs cytosol (n=823). Frequency distribution differed significantly between the two sets ($p < 1 \times 10^{-30}$, $x^2 = 196.34$, $df = 19$; Table S10). Relative usage frequency of amino acids Ala (A), Ser (S), Cys (C), Glu (E), Pro (P), Thr (T), Trp (W), Phe (F), Ile (I), Gly (G), Leu (L), Arg (R) differed significantly between the two sets (pair-wise Chi-square analysis; $p < 0.0001$ for A, T, F, I, L, R; $p < 0.01$ for S, E, P, W; $p < 0.05$ for C, G). (E) Relative frequency of P2 amino acid usage in proteins localized to nucleus (n=639) vs cytosol. Frequency distribution differed less significantly between the two sets ($p > 0.005$, $x^2 = 36.33$, $df = 19$; Table S10). Relative usage frequency of amino acids Ser (S), Cys (C), Asp (D), Leu (L) differed significantly between the two sets (pair-wise Chi-square analysis; $p < 0.001$ for S; $p < 0.01$ for C, D, L). (F) Estimated total relative abundance of proteins that are encoded with Ubr1-QC-compatible P2 residues by localization category. Abundance levels based on Huh et al., 2003.

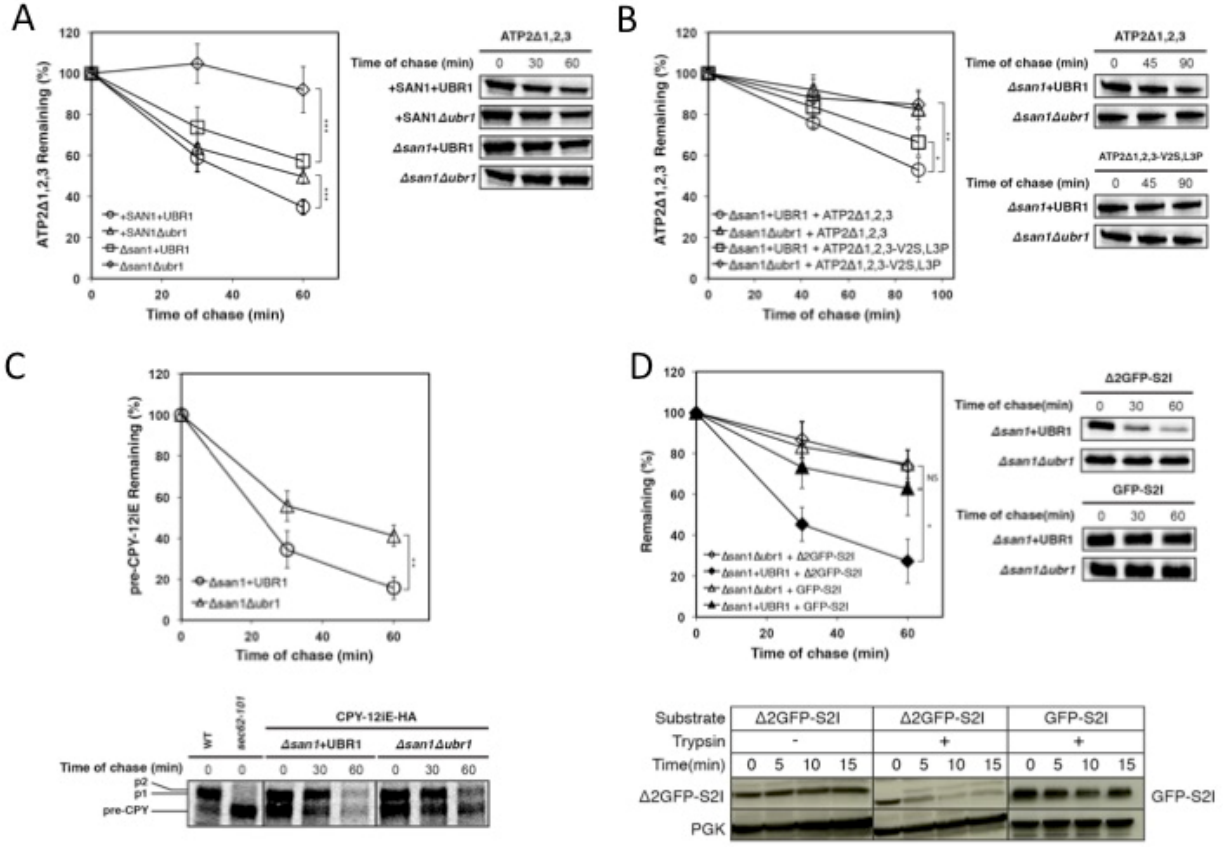

Figure 3. Mistranslocated forms of mitochondrial and secretory proteins are substrates of cytosolic quality control mediated by Ubr1 and the N-end rule pathway. Efficient recognition of CytoQC substrates by Ubr1 is reliant on a bi-partite degradation signal comprising of a Ubr1-QC-compatible P2 residue and structural aberrancy. (Figures adapted from Tran, 2013) (A) ATP2Δ1,2,3 turnover was determined by metabolic pulse-chase analysis in +SAN1+UBR1 (wild-type), Δsan1+UBR1, +SAN1Δubr1, and Δsan1Δubr1 strains. (B and C) The turnover of ATP2Δ1,2,3 , ATP2Δ1,2,3-V2S,L3P, or CPY-12iE-HA was determined by metabolic pulse-chase analysis in Δsan1+UBR1, and Δsan1Δubr1 strains. Samples from Δsan1Δubr1 cells expressing CPY-12iE-HA were exposed to a phosphor screen for an extended period (5 days vs 3 days for Δsan1+UBR1 cells) for imaging and quantitative analysis due to a lower overall detection level of the expressed substrate compared to Δsan1+UBR1 cells. (D) Turnover of GFP-S2I and Δ2GFP-S2I was compared by pulse-chase analysis in Δsan1Δubr and Δsan1+UBR1 cells. Trypsin sensitivity assay: postnuclear lysates were prepared from Δsan1Δubr cells and treated with 5.0μg/ml trypsin for the durations indicated. Protein was analyzed by immunoblotting with monoclonal anti-HA antibody. Endogenously expressed phosphoglycerate kinase (PGK) was assayed to serve as a folded protein control. (A-D) Metabolic pulse-chase: Cells were grown to log phase and shifted to 30°C for 30 minutes followed by pulse-labeling with [35S]methionine/cysteine for 10 minutes (ATP2, CPY-12iE-HA) and chased at the times indicated. Proteins were immunoprecipitated using anti-HA antibody and resolved by SDS- PAGE, then visualized by phosphoimager analysis. Error bars, mean +/- SD of at least three independent experiments. Student's t-test: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$; Not Significant (NS), $p > 0.5$.

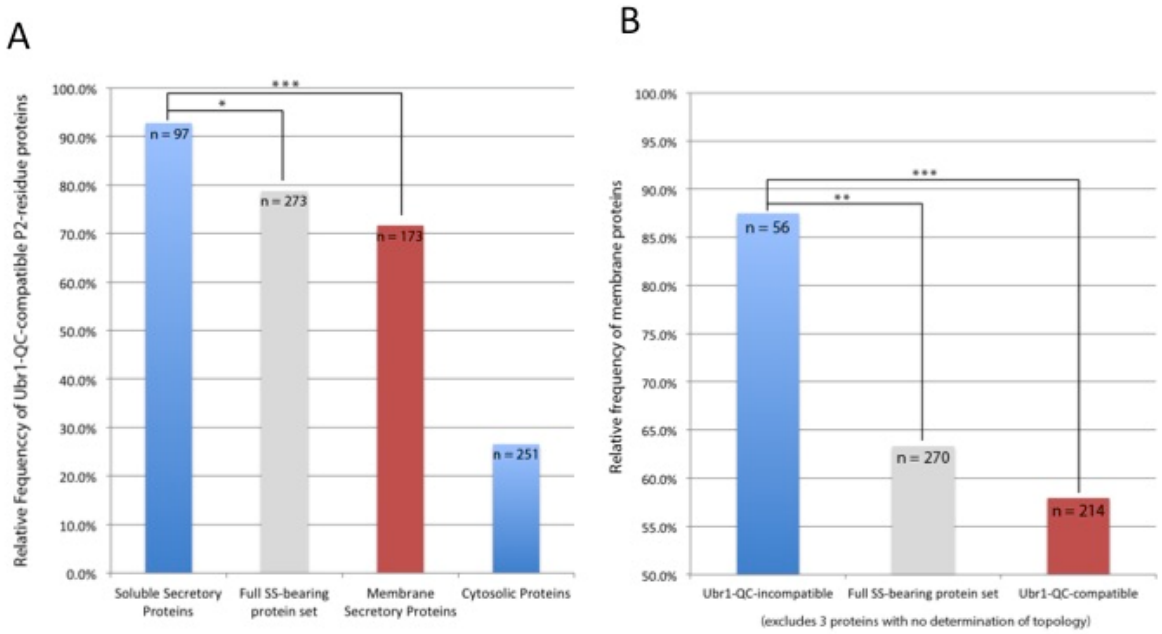

Figure 4. The vast majority secretory proteins encode Ubr1-QC-compatible P2 residues, while the vast majority of secretory proteins encoding Ubr1-QC-*incompatible* P2-residues are membrane proteins. (A) The percentages of proteins encoded with Ubr1-QC-compatible P2 residues was calculated for three categories of proteins derived from the full set of 273 signal-sequence bearing proteins: those determined to be soluble, membranous, and the full set (see Materials and Methods; Tables S12, S14A, S15A, S16). Chi-square test: Soluble vs full set: *, $p < 0.005$, $x^2 = 9.7$, $df = 2$. Soluble vs membrane set: ***, $p < 5 \times 10^{-5}$, $x^2 = 16.8$, $df = 2$. Cytosolic protein set (n = 251) from Forte et al., 2011. (B) The percentages of proteins that are membrane-bound was calculated for three categories derived from the full set of 270 signal-sequence bearing proteins for which topology was determined: those encoded with Ubr1-QC-compatible P2 residues, those encoded with Ubr1-QC-*incompatible* P2 residues, and the full set (see Materials and Methods; Tables S12,S14A, S15A, S16). Chi-square test: Ubr1-incompatible vs full set: **, $p < 0.001$, $x^2 = 11.7$, $df = 2$; Ubr1-incompatible vs Ubr1-compatible: ***, $p < 5 \times 10^{-5}$, $x^2 = 16.8$, $df = 2$.

MATERIALS AND METHODS

Note: All non-bioinformatic biological experimental methods described here are excerpted from the original thesis work performed by Anthony Tran at National University of Singapore for his doctoral dissertation (Tran, 2013). The original thesis work was performed in the lab of Associate Professor Davis Ng at the National University of Singapore.

Yeast strains used in this study are listed in Table S28. Anti-HA monoclonal antibody (HA.11) was sourced from Covance (Princeton, New Jersey). Monoclonal anti-3-phosphoglycerate kinase (PGK) was sourced from Invitrogen (Carslbad, California). Anti-CPY antibody was a gift from Reid Gilmore (University of Massachusetts, Worcester, MA). Anti-Gas1p antibody was raised against amino acids 40 to 289 of Gas1p (Davis Ng, NUS/TLL).

Plasmids and primers

Standard cloning procedures were utilized for the construction of plasmids (Sambrook, et al., 1989). Unless otherwise stated, exogenously expressed substrates possess an engineered single hemagglutinin (HA) epitope tag attached to the C-terminus. Ste6*C, Δ2GFP , ATP2Δ1,2,3, and their derivatives, were expressed under control of a high expression, constitutive TDH3 (glyceraldehyde- 3-phosphate dehydrogenase) promoter in yeast centromeric plasmids. HA-tagged Prc1p (CPY/carboxypeptidase Y) and its derivatives were placed under control of its native constitutive endogenous promoter in yeast centromeric plasmids. Site-directed mutagenesis of the original constructs expressing Ste6*C, Δ2GFP, and ATP2Δ1,2,3 was performed to generate mutant substrates.

pAT1: A fragment carrying the PRC1 promoter was PCR amplified from pSW119 with BamHI and NotI restriction ends. The amplified fragment and pSW119 were digested with NotI and BamHI and ligated to generate pAT1.
pAT32: A fragment encoding the TDH3 promoter, followed by Ste6*C-HA, followed by the ACT1 terminator sequence, was PCR amplified from pRP22 with primers AT270 and AT273 and digested with NotI and XhoI. The fragment was then ligated into an empty pRS316 vector to generate pAT32.
pAT33-pAT51: pAT33 through pAT51 (expressing Ste6*C-I2K, Ste6*C-I2Y, Ste6*C-I2F, Ste6*C-I2A, Ste6*C-I2L, Ste6*C-I2E, Ste6*C-I2V, Ste6*C-I2G, Ste6*C-I2R, Ste6*C-I2M, Ste6*C-I2P, Ste6*C-I2W, Ste6*C-I2N, Ste6*C-I2D, Ste6*C-I2H, Ste6*C-I2Q, Ste6*C-I2C, Ste6*C-I2T, Ste6*C-I2S) were constructed by mutation of the base-pairs encoding the 2nd residue of Ste6*C through site-directed mutagenesis using primers AT21-AT39 and pRP22 as a template.
pAT52: A fragment encoding residues 1201-1290 of the STE6 ORF followed by a hemagglutinin epitope (HA-tag) sequence was PCR amplified from yeast genomic DNA using

primers AT40 and AT41. The fragment was digested with BamHI and XbaI and ligated to pRP22 digested with BamHI and XbaI generating pAT52.
pAT55-pAT57: pAT55 through pAT57 (expressing Δ2GFP-S2I, Δ2GFP-S2F, Δ2GFP-S2K) were constructed by mutation of the base-pairs encoding the 2nd residue of Δ2GFP through site-directed mutagenesis using primers AT18-AT20 and pRP44 as a template.
pAT61: A 741bp fragment of the GFP ORF followed by the hemagglutinin (HA) tag sequence was PCR amplified using mutational primers AT189 and AT190 and pAT7 as a template. The resultant PCR product carrying the GFP ORF with the 2nd residue mutated to an isoleucine was digested with BamHI and XbaI and ligated to pAT7 digested with BamHI and XbaI generating pAT61.
pAT64: A 1563-bp fragment carrying the ATP2 ORF followed by the hemagglutinin epitope (HA-tag) sequence was PCR amplified from yeast genomic DNA using primers AT244 and AT226. The fragment was digested with BglII and XbaI and ligated to pAT7 digested with BamHI and XbaI generating pAT64.
pAT65: pAT66 was digested with ClaI and XhoI to release a 2671-bp fragment encoding the TDH3 promoter, ATP2Δ1,2,3-HA, and ACT1 terminator sequences. This fragment was ligated into an empty pRS316 vector digested with ClaI and XhoI to generate pAT65.
pAT66: A 1506-bp fragment carrying the ATP2 ORF with deletions of residues 5- 12, 16-19, and 28-34, followed by the hemagglutinin epitope (HA-tag) sequence was PCR amplified from yeast genomic DNA using primers AT246 and AT226. The fragment was digested with BglII and XbaI and ligated to pAT7 digested with BamHI and XbaI generating pAT66.
pAT68: pAT68 (expressing ATP2Δ1,2,3-V2S,L3P-HA) was constructed by mutation of the sequences encoding the 2nd and 3rd residues of ATP2Δ1,2,3-HA, from valine to serine at the P2 position, and leucine to proline at the 3rd position, through site-directed mutagenesis using primer AT275 and pAT66 as a template.
pAT72: pAT72 (expressing PRC1-12iE) was constructed by the insertion of a three base-pair sequence encoding a glutamic acid residue at the 12th codon position of the PRC1 ORF through site-directed mutagenesis using primer AT280 and pXW92 as a template.

Metabolic Pulse-Chase Assay

Yeast cells were grown to log phase at 30°C (25°C for temperature sensitive strains). 3 OD_{600} units of cells were resuspended in 0.9ml of SC or SC selective media and incubated at 30°C (37°C for temperature sensitive strains) for 30 minutes. Pulse labeling was then initiated with the addition of 82.5 μCi of [35S]Met/Cys (EasyTagTM EXPRESS 35S, PerkinElmer) for 5 or 10 minutes depending on the labeling efficiency of the substrate of interest. Label was chased with the addition of excess cold methionine and cysteine to a final concentration of 2mM. At the appropriate timepoints, pulse labeling/chase was terminated by the addition of 100% TCA to a final concentration of 10%. Immunoprecipitation of samples and resolution by SDS-PAGE were carried out as described (Vashist *et al*., 2001). Phosphor screens exposed to gels (1 to 5 day

exposure, depending on substrate expression level) were scanned with a Typhoon™ phosphoimager and the visualized bands of interest quantified using ImageQuant TL software (GE Healthcare Life Sciences, Uppsala, Sweden). Background signal from screen exposure was subtracted. All results presented are the mean ± SD of at least three independent experiments.

Trypsin Sensitivity Assay

Yeast cells expressing the substrate of interest were grown to log phase (0.4-0.6 OD) and resuspended in cytosol buffer (20 mM HEPES-KOH, pH 7.4, 14 % glycerol, 100 mM KOAc, and 2 mM MgOAc) at a concentration of 20 OD/mL. 1mL of this resuspension was transferred to a 2ml screw-cap tube and homogenized by vortexing for 30 seconds in the presence of 1ml of 0.5mm diameter zirconium beads followed by a 1 minute incubation at 4°C. This was performed for 5 cycles. The homogenate was transferred to 1.5ml eppendorf tubes. 0.6ml of fresh cytosol buffer was used to wash the beads and pooled with the original homogenate. Post-nuclear lysate was isolated by pelleting at 500xg for 5 minutes and transferring the supernatant to a fresh tube. The post-nuclear lysate was incubated at 30°C for 5 minutes, followed by the addition of trypsin to a concentration of 5µg/ml. Samples were vortexed and incubated for 30°C, with 100ul aliquots taken at the indicated timepoints and mixed with 11.1ul of 100% TCA in fresh 1.5ml eppendorf tubes. Aliquots were kept on ice for 5 minutes and pelleted at 14000rpm for 20 minutes at 4°C. Supernatant was discarded, sample pelleted again briefly, and supernatant again discarded. The pellet was resuspended in 10ul of TCA resuspension buffer (100 mM Tris-HCl pH 11.0, 3 % SDS, 1mM PMSF) by cycles of boiling at 100°C and vortexing. Samples were pelleted at 4°C to remove SDS and other insoluble particles, and the resultant supernatant transferred to a fresh tube. Analysis by SDS-PAGE/Western Blotting was performed using the appropriate antibodies.

Edman Degradation N-terminal Seqeuncing

Yeast cells expressing the protein of interest were grown to 1 OD/mL in selective media. 800OD of yeast cells were harvested at 3000xg for 15 minutes, washed once with 1x PBS, and pelleted again at 3000xg. Cells were washed in IP/NP40/PIC/DTT (50mM Tris-HCl, pH8 150mM NaCl, 0.1% NP-40, 0.1mM DTT), pelleted at 3000xg, and resuspended in IP/NP40/DTT containing protease inhibitors (complete, mini, EDTA-free protease inhibitor cocktail tablet, Roche) at a concentration of 50 OD/ml. 1 mL aliquots of the resuspension were transferred to a 2ml screw-cap tubes and homogenized by beadbeating for 30 seconds using a Mini-BeadBeater cell disrupter (Biospec Products) followed by a 5 minute incubation on ice; beadbeating and incubation on ice was repeated for 6 cycles in the presence of 1ml of 0.5mm diameter zirconium beads. The homogenate was transferred to 1.5ml eppendorf tube. 0.6ml of fresh cytosol buffer was used to wash the beads and pooled with the original homogenate. Lysate was cleared by centrifugation at 14000 rpm and transferred to a fresh tube. Lysate was incubated with 65uL of Roche Anti-HA affinity matrix per 4mL of lysate for 2 hours or overnight at 4°C. Affinity matrix

was spun down at 2700rpm for 1 minute and washed with ice cold IP/NP40/PIC/DTT three times, followed by one wash with cold IP buffer to remove residual NP40. Bound proteins were eluted from matrix through the addition of protein loading buffer (PLB) and subsequent boiling at 100°C for 10 minutes. Immunoprecipitated proteins were resolved on 12% SDS-PAGE gels and transferred to a PVDF membrane. Membrane was washed with dIH2O for 1 minute (3 x 1 minute, shaking at 70rpm) to eliminate traces of SDS, Tris, glycine, and other reagents that have the potential to interfere with Edman chemistry. The membrane was then stained with Coomassie Brilliant Blue (0.1% CBB, 5% acetic acid, 50% methanol) for 5 minutes by shaking at 70rpm. Membrane was quickly destained with 50% methanol (3 x 1 minute, shaking at 70rpm). Band containing the protein of interest was excised from the membrane and cut into smaller pieces to facilitate sample analysis. Membrane fragments were loaded into an ABI Procise 494 Sequencer for sequencing using standard manufacturer recommended protocols.

Bioinformatic analysis

The raw data file containing protein translations for systematically-named ORFs was obtained from Saccharomyces Genome Databse (SGD) . To facilitate parsing with PHP, quotation marks in protein descriptions were removed, and a termination character ("@") was added to the end of the file. (http://downloads.yeastgenome.org/sequence/S288C_reference/orf_protein/; orf_trans.fasta.gz) A PHP script was written to extract from this data file the systematic name of each ORF and the first 2 residues in its respective protein sequence, and subsequently output into an SQL database; a list of 5887 ORFS and their N-terminal sequence were produced (Table S1). The list of proteins exclusively localized to each of the main protein localization categories (nuclear, mitochondrial, cytoplasmic, or secretory) was determined based on complete or partial localization to that category, as determined by the localization terms assigned to each protein through a GFP-fusion localization method (Huh et al., 2003). Nuclear proteins included those assigned the following localization terms: *nucleus, nucleolous,* and *nuclear periphery*. Secretory proteins included those assigned the following terms: *ER, Golgi, vacuole, endosome,* and *peroxisome*. Proteins assigned the term *mitochondrion* were categorized as mitochondrial. Proteins assigned with the term *cytoplasm* were categorized as cytosolic. To determine the relative total abundance of proteins with nuclear, cytosolic, secretory, or mitochondrial localization that encode Ubr1-QC-compatible or Ubr1-QC-incompatible P2 residues, we summed the abundance levels of proteins in each subset as quantified in the GFP-localization study (Huh et al. 2003), and divided it by the aggregate abundance of all proteins within the full localization category (Table S17-S27).

Categorization of signal-sequence bearing proteins

A published set of 277 signal-sequencing bearing proteins was analyzed (Table S12; Forte et al., 2011). Two of this original set were duplicate entries (AIM6/YDL237W duplicate of LRC1,

FLO11/YIR019C duplicate of MUC1) and were not included in the analysis. Two were not present in the YeastSGD database and were also excluded (YCR012C, and YJL052C) (Table S13). After these exclusions, a manual review was performed on each protein to identify literature that supported soluble or membranous topology. 211 of the proteins were successfully categorized through the review of literature (Tables S14-S16, S14A, S15A). For 59 proteins for which supporting literature could not be found, protein sequences of each were analyzed with SignalP 4.1 using the default optimized parameters. Proteins that were not predicted to possess a signal sequence cleavage site were categorized as membrane proteins. To assess the topology of proteins that were predicted to possess a signal sequence cleavage site, Kyte-Doolittle hydropathy profiles were obtained, and membrane prediction with Phobius performed. Kyte-Doolittle analysis was performed with a window size of 19. Proteins with a hydrophobicity score of greater than 1.8 after the predicted cleavage site were categorized as membrane proteins. Proteins that did not have a hydrophobicity score of greater than 1.8 after the predicted cleavage site were categorized as soluble proteins. 3 proteins which did not have consensus between Kyte-Doolittle and Phobius analysis were excluded from relative frequency calculations involving topology.

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request, or are included in the supplemental tables provided.

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SUPPLEMENTAL DATA

See PDF and Excel files for Supplemental Tables.

Supplemental Figures S1-S6 (All figures adapted from Tran, 2013)

Edman's Degradation sequencing of Ste6*C-I25 isolated from Δsan1Δubr1 cells

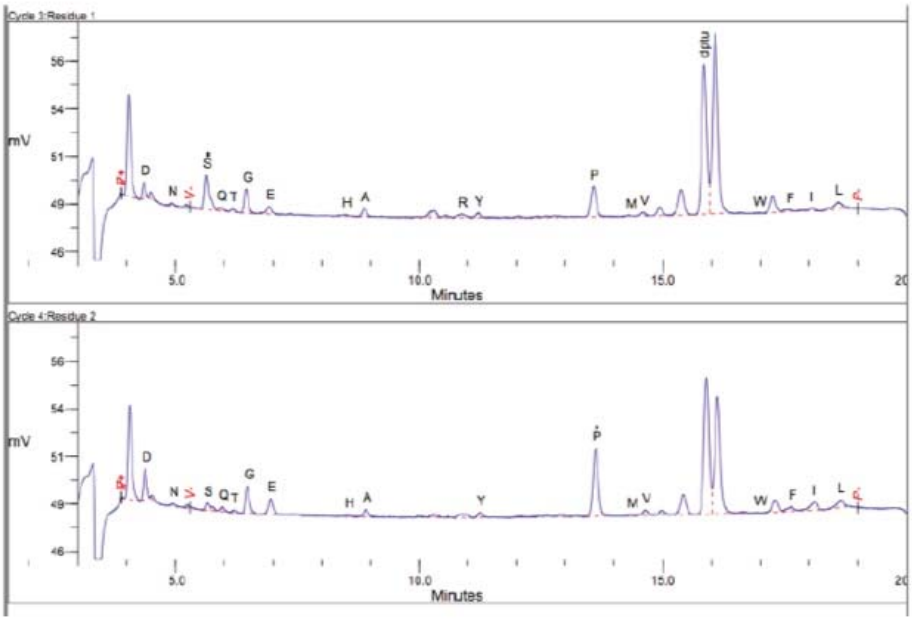

Figure S1A

Edman's Degradation sequencing of Ste6*C isolated from Δsan1Δubr1 cells

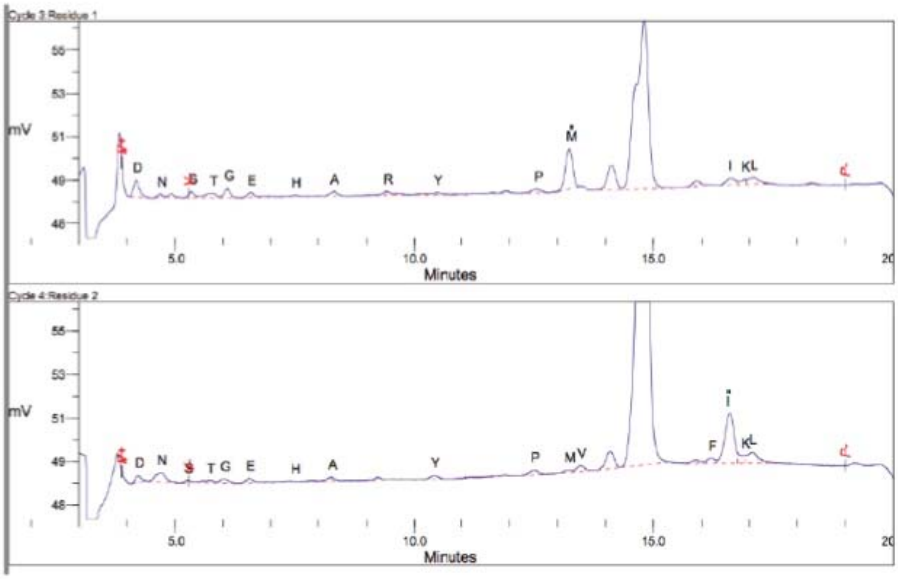

Figure S1B

Edman's Degradation sequencing of Ste6*C-I2V isolated from Δsan1Δubr1 cells

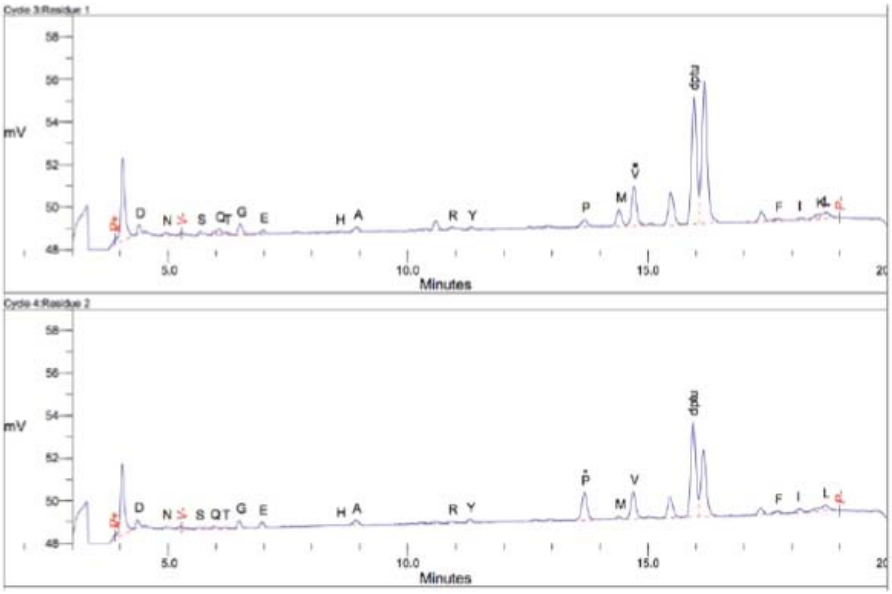

Figure S1C

Figure S1. N-terminal sequencing of Ste6*C constructs. (A) Lysate was prepared from Δsan1Δubr1 cells expressing Ste6*C-HA. Protein was immunoprecipitated from lysate with anti-HA affinity matrix (Roche), resolved by SDS-PAGE, and transferred to PVDF membrane. Protein band carrying Ste6*C-HA was excised from the membrane and sequenced via Edman degradation. (B) Lysate was prepared from Δsan1Δubr1 cells expressing Ste6*C-HA-I2S. Protein was immunoprecipitated from lysate and sequenced as described in Figure S1A. (C) Lysate was prepared from Δsan1Δubr1 cells expressing Ste6*C-HA-I2V. Protein was immunoprecipitated from lysate and sequenced as described in Figure S1A.

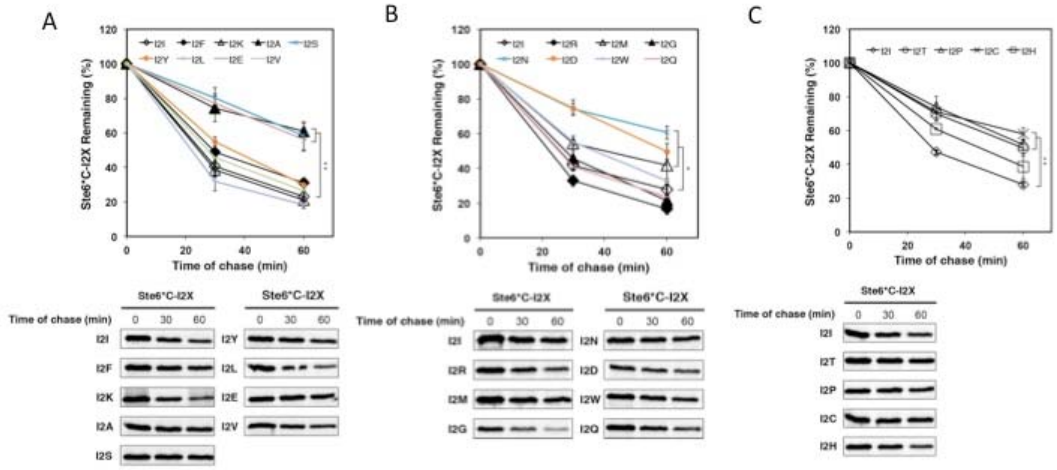

Figure S2

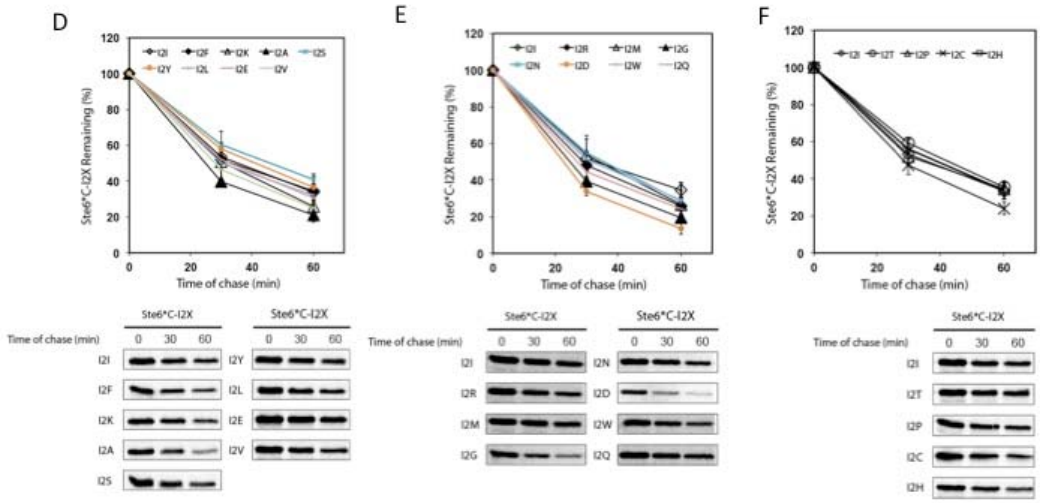

Figure S2

Figure S2. Efficient degradation of Ste6*C by the Ubr1p quality control pathway is highly dependent on P2 residue identity. (A), (B), and (C) Turnover rates of nineteen Ste6*C P2 amino acid residue mutants (Ste6*C-I2X, where X = A[alanine], N[asparagine], S[serine], C[cysteine], E[glutamic acid], P[proline], D[aspartic acid], T[threonine], M[methionine], H[histidine], W[tryptophan], F[phenylalanine], Y[tyrosine], V[valine], Q[glutamine], G[glycine], K[lysine], L[leucine], R[arginine]) was compared to the turnover rate of the original Ste6*C substrate (Ste6*C-I2I) in Δsan1+UBR1 background cells by pulse chase analysis as described in Figure 1A. (D), (E) and (F) Same as described in S2A, but in +SAN1Δubr1 background cells. Error bars, mean +/- SD of at least three independent experiments.

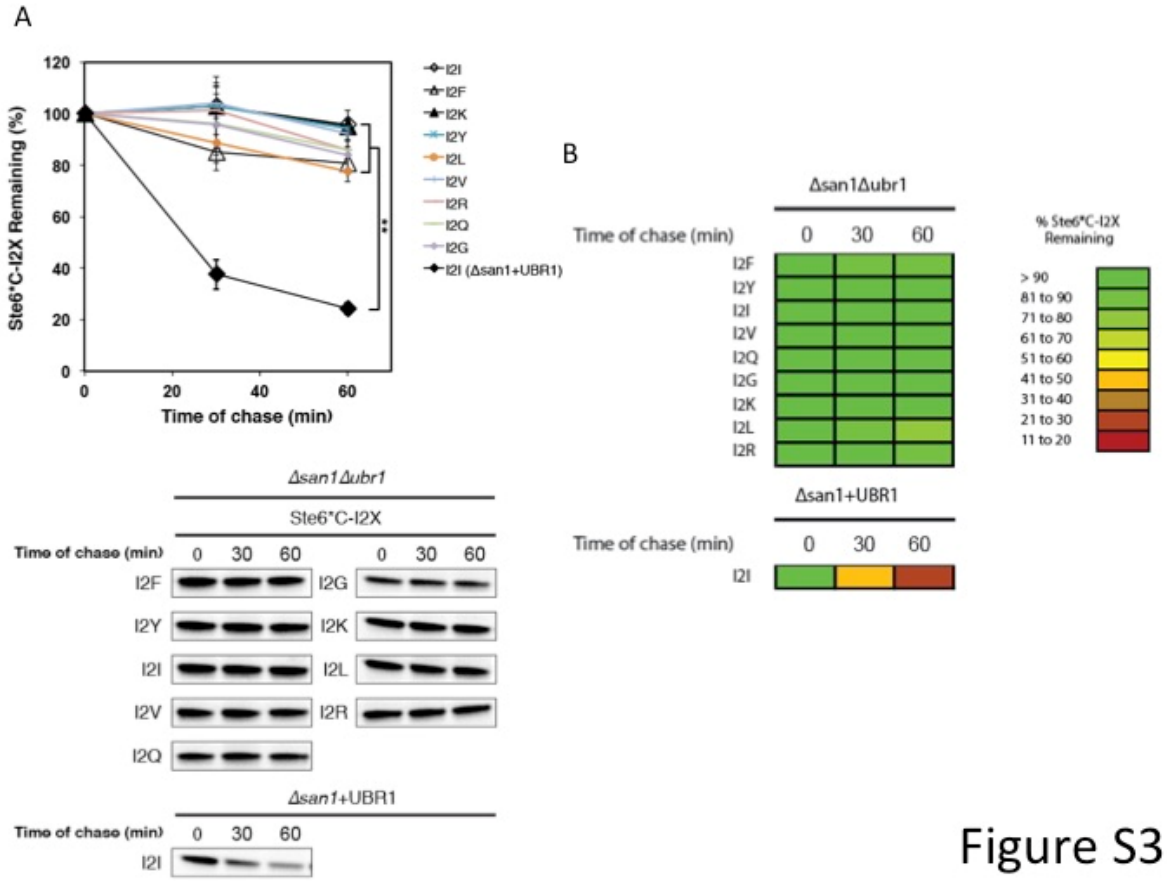

Figure S3. Ste6*C-I2X mutants bearing destabilizing P2 residues are stabilized in Δsan1Δubr1 cells. (A) Turnover rates of N-terminally destabilized Ste6*C-I2X mutants in Δsan1Δubr1 cells were assessed by pulse-chase analysis as described in Figure 1A. Degradation of Ste6*C-I2I in Δsan1+UBR1 cells was also examined as a control. Error bars, mean +/- SD of three independent experiments. (B) Heat map reflecting turnover rates of Ste6*C-I2X substrates in Δsan1Δubr1 as presented in S3A.

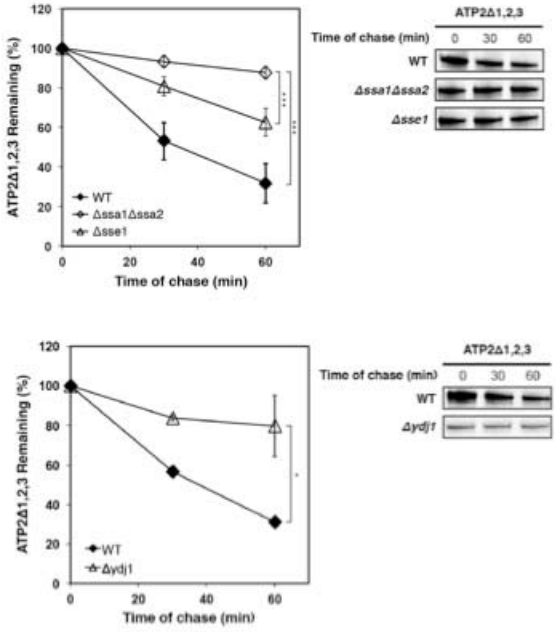

Figure S4

Figure S4. Degradation of ATP2Δ1,2,3 is dependent on chaperones involved in CytoQC degradation. Turnover of ATP2Δ1,2,3 in WT, Δydj1, Δsse1Δsse2, and Δssa1 cells was analyzed by pulse-chase as described in in 5A. Student's t-test: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$; Not Significant (NS), $p > 0.5$. Error bars, mean +/- SD of at least three independent experiments.

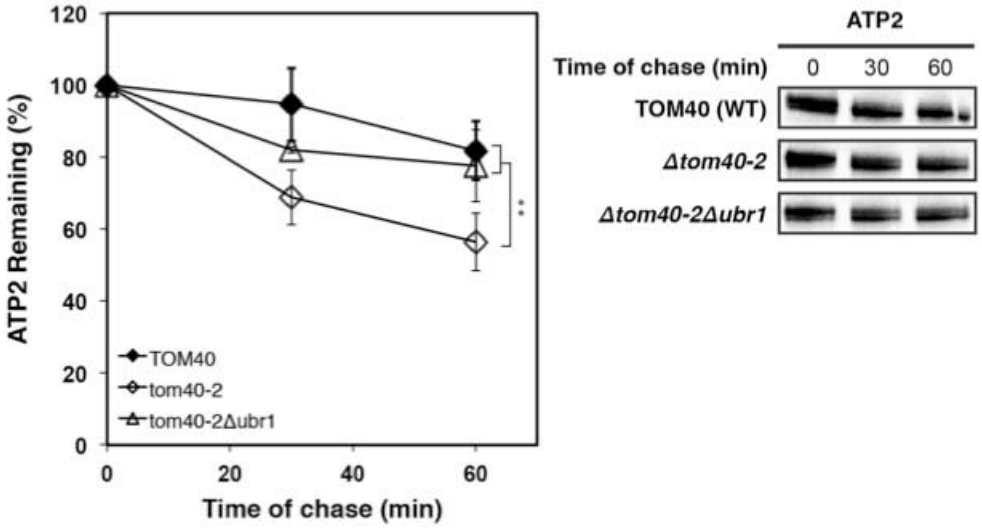

Figure S5

Figure S5. Wild-type Atp2p is degraded by Ubr1p in mitochondrial import mutant strain *tom40-2*. HA-tagged wild-type ATP2 protein expressed in TOM40 (wild-type), tom40-2, and tom40-2Δubr1 cells was analyzed by pulse-chase analysis as described in Figure 1A. Error bars, mean +/- SD of at least three independent experiments.

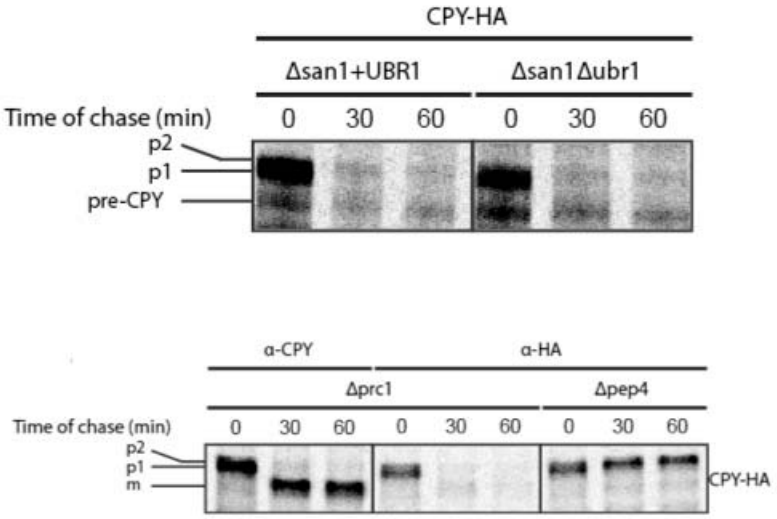

Figure S6

Figure S6. C-terminal HA-tag is removed from HA-tagged CPY in the vacuole. Pulse-chase analysis of CPY-HA was performed in Δprc1, Δpep4, Δsan1+UBR1, and Δsan1Δubr1 cells as described in Figure 1A. CPY was probed with anti-CPY and anti-HA antibodies in Δprc1 cells, and anti-HA in Δpep4 cells. Error bars, mean +/- SD of at least three independent experiments.